NIAMDD ANNUAL REPORT

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U.S. National Institute of Arthritis, Metabolism, and Digestive Diseases
" Report of program activities

NIAMDD

ANNUAL PROJECT REPORTS

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Project Reports

ANNUAL REPORT SUMMARY
LABORATORY OF NUTRITION & ENDOCRINOLOGY
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM & DIGESTIVE DISEASE

PILOT PLANT UNIT

Large-Scale Processing of Biological Materials

During the past year, a total of 260 requests were processed for investigators: NIAMDD 122; BoB 42; NICHD 32; NCI 16; NHLBI 13; NIMH 8; NIDR 7; NINCDS 7; NIAMID 3; NEI 1; and others 8. 184.5 kg quantities (42,590 liters of cultures) of 18 organisms were produced. The microorganisms grown during this period include wild type and mutant strains of *Arthrobacter luteus, Bacillus amyloliquefaciens, Bacillus pumilis, Bacillus stearothermophilus, Bacillus subtilis, Bordetella pertussis, Clostridium pasteruianum, Escherichia coli, Euglena gracilis, Haemophilus influenzae, Haemophilus parainfluenzae, Klebsiella cloaceae, Neisseria meningitidis, Pneumococcus sp., Pseudomonas sp., Saccharomyces cerevisiae, Salmonella typhimurium, Streptococcus mutans*. Some of the organisms were used for the production of bacteriophages MS2, lambda, T_4 and T_7, others were used to produce extracellular enzymes. Microorganisms were grown 68 times in the two 10-liter, 70 times in the 50-liter, 62 times in the 300-liter and 18 times in the 1100-liter fermentors. Of the 200 fermentations, 23 (11.5%) were contaminated with 18 of these traced to the inocula supplied by the requesting investigator.

Studies were continued with the Division of Bacterial Products, Bureau of Biologics for polysaccharide production by *H. influenzae* and isolation of the enterotoxin proteins from special strains of *E. coli*. Spheroplasts were produced for the NHLBI from *E. coli* using 10-, 50-, and 300-liter fermentors in series. Some of the spehroplasts were recovered in the new Electro-Nucleonics RK zonal centrifuge in an attempt to improve the quality of the product. The Gaulin laboratory homogenizer was used 45 times to rupture cell suspensions of yeast, protozoa or bacterial cells.

Large scale processing activities consisted of the isolation of polynucleotide phosphorylase from 1 kg (dry) *Micrococcus lysodeiktieus* cells for NCI; the preparation of Exonuclease-5 from 3.6 kg *E. coli*, ATPase from a 20-liter culture of *Acanthamoeba castellanii* and preliminary isolation studies for the extraction of enzymes from chicken livers using the new RK centrifuge for NHLBI; the fractionation of 18.6 liters equine meningococcal group B antiserum and preparation of IgG component from 18.3 liters of burre cholera toxin antiserum for the BoB; the isolation from two 1100-liter cultures of *B. amyloliquefaciens*, the enzyme Barnase, and its inhibitor, Barstar, from 37 kg of cells and the preparation of three two-gallon quantities of hydroxylapatite for NIAMDD. Various plant and animal tissues were either dried, and/or ground in the large ovens, mills and related equipment and solutions were centrifuged such as 30 liters of Killam rat virus and several 15-liter cultures of special *E. coli* mutants. Large volumes of supernatants were concentrated including the lyophilization of 9 liters to dryness in the Stokes freeze-dryer, 8 liters to 700 ml in the circulating evaporator and 1100 liters to 25 liters in the Pfaudler reactor.

GERM-FREE RESEARCH UNIT

Studies in Experimental Nutrition.

Studies of the production of tumors in rats by cycasin and related compounds have been continued. Two precursors (dimethyl hydrazine and azoxymethane) of the aglycone of cycasin are carcinogenic in both germfree and nongermfree rats. The location of the tumors observed with these two compounds differ from those produced by the aglycone itself, indicating that the compounds are intrinsically active or are converted to active compounds other than the aglycone.

Maintenance of normal levels of plasma vitamin A, in addition to being dependent on adequate amounts of zinc, is related also to growth of the animal and/or dietary intake. Lack of growth resulted also in below normal levels of plasma zinc. Maintenance of normal levels of plasma zinc can be achieved in the absence of growth by intraperitoneal administration of rather large daily doses of zinc. Preliminary results indicate that the plasma vitamin A may be increased even in the absence of growth when the plasma zinc is maintained at elevated levels. (J. Smith, VA Hospital, and E. G. McDaniel).

DEVELOPMENTAL BIOCHEMISTRY SECTION

Composition and Structure of the Nucleosome, the Fundamental Repeating Subunit of Chromatin.

1) With the use of specific anti-histone antibody and sedimentation of the products, isolated nucleosomes have been shown to contain two molecules each of the four smaller histones.

2. Nucleosomes melt in an all-or-none fashion. Histone cross-linking experiments, controlled by similar modification not introducing covalent cross-links, demonstrate that histone-histone dissociation is a prerequisite for DNA strand separation during thermal denaturation. Chemical modification of chromatin with acetic anhydride-^{14}C and determination of the specific radioactivity of several lysyl residues, localized within the known primary sequence of the four smaller histones after tryptic digestion and fingerprinting, is consistent with models suggesting that the carboxyl terminal portions of the histones interact with one another to form a nucleosome core; the amino terminal portions of the histones bind to DNA wound around the protein nucleus.

3) The basic repeat distance along the DNA between initial cleavages has been defined as 200 base pairs, and it has been possible to show that the nucleases used for nucleosome preparation then nibble down this initially cleaved DNA to a fragment size of 140 to 160 base pairs. This suggestion that within the 200 base pair repeat there is a region of 40-50 base pairs in a more nuclease susceptible conformation is reinforced by the demonstration that histone H_1 only affects the digestion properties of this DNA. Presumably H_1 is bound to the DNA bridges between nucleosomes.

4) The 160 base pair length of DNA within the nucleosome can be nicked at ten base intervals by endogenous endonuclease of rodent liver or DNase I, while the bridging DNA does not appear to be susceptible to this type of degradation.

The 5' termini of the nucleosome DNA can be labeled with ^{32}P using ATP and polynucleotide kinase. Digestion of this DNA and autoradiography of the electrophoretically separated single stranded DNA fragments allows assignment of favored and blocked sites for nicking by DNAse I within the linear sequence of DNA in the nucleosomes.

5) Homogeneous monomeric nucleosomes have been prepared and their properties compared with those of chromatin prepared by both classical methods and the nuclease digestion method. The major difference is the circular dichroism spectrum: maximal ellipticity for nucleosomes is 1900° compared to 4500-5000° for chromatin and 10000° for DNA. Urea alters the physical properties of nucleosomes in the same fashion as it does bulk chromatin. Nucleosomes are bound by DNA-dependent RNA polymerase but the rate of transcription of the histone bound particle appears to be very slow. (J. P. Whitlock, Jr., L. C. Tack, M. Bustin, and R. T. Simpson).

Purification of the Restriction Endonuclease BAM I

The enzyme has been purified free of other DNase, exonuclease, and phosphatase activities. It and other restriction endonucleases are being utilized to prepare large amounts of defined length, sequence homogeneous oligonucleotides for use in characterization of histone-DNA interactions in nucleosomes (G. W. Rushizky and J. H. Mozejko).

Binding of Triiodothyronine to Chromatin

A reproducible method of assessing the binding of triiodothyronine to chromatin has been developed. Using low concentrations of Triton X-100 and hydroxyapatite to adsorb the chromatin, saturable binding is obtained at a level corresponding to about 1000 molecules of bound hormone per haploid equivalent for liver chromatin. The localization of hormone binding sites in template-active and repressed chromatin segments and attempted isolation of regulatory proteins and/or DNA sequences which interact with triiodothyronine are in progress. (D. Gruol and R. T. Simpson).

Isolation of Hela Cell Nuclei

Methods have been developed for the isolation of HeLa cell nuclei under non-aqueous conditions. The concentrations of DNA, RNA, protein, Na K, Ca, and Mg have been determined in such nuclei. Full elucidation of the ionic composition of the mammalian nucleus is in progress. (S. Yamazaki and R. T. Simpson).

Studies of Protein-Protein Interactions.

Small octahedral crystals of the complex of B. amyloliquefaciens ribonuclease (barnase) with its stoichiometric protein inhibitor have been grown. Complementation studies with barnase fragments have shown that a C-terminal 23 residue peptide binds to barnase lacking the C-terminal 8 residues with a dissociation constant of 6×10^{-12} M and restores 60% of native enzymatic activity (R. W. Hartley).

Biochemistry of Pituitary Glycoprotein Hormones

The subunits of bovine TSH have been separated and purified by an improved two stage gel electrophoresis procedure. During studies designed to improve the conditions for subunit preparations it was discovered that certain preparations of TSH contain nicked peptide chains which, upon SDS gel electrophoresis yielded low molecular weight fragments. Such findings suggest caution in the interpretation of subunit function and the nature of the bands found with SDS gel electrophoresis. (P. Condliffe and K. Sorimachi).

MEMBRANE REGULATION SECTION

A. An Allosteric Model for the Actions of Glucagon and Guanine Nucleotides on Adenylate Cyclase.

We have published previously a model for hormone and guanine nucleotide action in which it was proposed that the enzyme system exists in three states of activity having different affinities for protonated ATP, postulated to be a potent inhibitor of the enzyme. Although the data could be fitted satisfactorily to this model, the complexity of the model and the very indirect evidence for the putative inhibitory role of protonated substrate compelled us to seek for other models that could be tested. Recently, a two state, allosteric model was tested with the hepatic enzyme system. This model proposes that the enzyme exists in an inactive state (A) in equilibrium with an active state (B) and that the various ligands that increase enzyme activity, such as glucagon and guanine nucleotides (GTP or Gpp(NH)p), do so by interacting preferentially with the active form of the enzyme. This model was tested by conventional computer fitting processes with data obtained by varying the concentrations of the activating ligands in various combinations. Satisfactory fits were obtained which allowed computation of the relative affinities of glucagon, GTP, and Gpp(NH)p for each state, GTP and glucagon interact preferentially to the B versus A state by a factor of 5- and 9-fold, respectively, whereas Gpp(NH)p binds with a 100-fold preference to the active state. The model assumes that changes in activity of the enzyme reflect both a modification of the structure of the enzyme system and conformational changes at the active site which alter the catalytic rate constant. As will be discussed below, factors that appear to influence the catalytic rate constant are metal ions (Mg and Mn) and adenosine. The simplicity of the model does not, of course, indicate that the processes involved in the activation process are really simple since it is now reasonably certain that three components, receptor, guanine nucleotide regulatory component, and catalytic component participate in the activation process. Nevertheless, the allosteric model is a means of testing, in a relatively straightforward manner, the characteristics of the system in response to the numerous ligands known to alter the activity of adenylate cyclase. (G. G. Hammes and M. Rodbell).

B. The Actions of Divalent Cations on Hepatic Adenylate Cyclase

The effects of divalent cations (Mg, Mn, and Ca) on hepatic adenylate cyclase have been investigated with the view that the cations may interact with sites on the enzyme that increase the catalytic breakdown of ATP to cyclic AMP. The following evidence supports the existence of these sites:

1. MgATP and MnATP display identical affinities for the active site.

2. Mn increases catalytic activity when added in the presence of MgATP; based on finding (1), this effect of Mn cannot be explained by an action at the catalytic site.

3. The stimulatory effect of Mn is independent of the concentration of ATP, indicating that its effect is not due to alterations in the concentration of free (or protonated) ATP as an inhibitor.

4. Pretreatment of the enzyme system with Gpp(NH)p, which causes the formation of a persistent activated state, in combination with Mg or Mn results in enhanced activity of the enzyme over that seen with Gpp(NH)p alone. After washing such pretreated membranes and assaying for enzyme activity, the stimulatory effects of added metal ions (over that required for complexing ATP at the active site) are either no longer observed or are attenuated.

5. Calcium ion has complex effects: It inhibits, pseudo competitively, the stimulatory effects of Mg; it stimulates Gpp(NH)p activation during pretreatment; it forms complexes with ATP which seem not to be substrates at the catalytic site; it is a weak inhibitor in the presence of Mn^{2+}. These findings suggest that calcium ion may interact at several sites on the enzyme system.

6. Mn^{2+} has a 10- to 15-fold greater affinity for the metal ion site(s) than does magnesium ion.

7. Mn^{2+} is a poor activating ligand in the presence of glucagon whereas the cation causes marked activation in the presence of Gpp(NH)p. These findings suggest either Mn^{2+} has more than one site on the enzyme system or that glucagon and Gpp(NH)p produce states of the enzyme that are affected differently by the cation. (C. Londos and M. Preston).

C. Regulation of Hepatic Adenylate Cyclase Activity by Adenosine

Adenosine affects the production of cyclic AMP in several types of cells; in some cells, the nucleoside increases production, in others it inhibits. The mechanism of these effects is unknown. The following evidence indicates that adenosine reacts directly with the hepatic adenylate cyclase system and plays an important regulatory role in the actions of metal ions, glucagon, and guanine nucleotides:

1. When added to the assay medium, adenosine inhibits basal activity and activity stimulated by Gpp(NH)p or glucagon.

2. The apparent K_i for adenosine inhibition is affected by Mg^{2+} and Mn^{2+} in proportion to the affinity of the cations for the metal ion activating site(s); thus the K_i with Mn^{2+} is ten-fold lower than observed with Mg^{2+} at sub-optimal concentration.

3. Glucagon and Gpp(NH)p cause a marked increase in the affinity of adenosine for the inhibitory site; this effect is particularly noted in the presence of Mg^{2+} and Mn2+.

4. The effect of adenosine is highly specific; numerous other purine and pyrimidine nucleosides tested fail to inhibit, and even seemingly minor modifications of the adenine ring led to loss of activity. The exception is 5'AMP

which is converted to adenosine by 5'-nucleotidase present in the plasma membrane. Moreover, adenosine deaminase abolishes the effects of adenosine. This effect of adenosine deaminase provides a useful tool for determining whether adenosine is present as a contaminant in the assay medium or is formed during the assay.

5. Finally, adenosine and divalent cations seem to be functionally linked in their actions on the enzyme in the sense that metal ions appear to promote the affinity of the system for adenosine and vice versa. (C. Londos and M. Preston).

Solubilization and Attempted Purification of the Adenylate Cyclase Components

Hepatic membranes have been dispersed with several detergents and the resultant "soluble" adenylate cyclase and guanine nucleotide binding proteins have been subjected to a variety of chromatographic procedures in an attempt to purify the components of the enzyme system. Adenylate cyclase subject to Gpp(NH)p-activation can be separated from about 60-70% of the membrane protein on Sepharose-6B columns provided that small amounts of detergent are included in the elution fluid. However, adenylate cyclase is associated with numerous proteins in the included fraction; efforts to separate adenylate cyclase from these proteins by a variety of techniques have proved unsuccessful thus far either because each of the proteins have identical properties, are aggregated in some type of complex, or are included in detergent micelles having nearly identical charge, size, and hydrodynamic properties. Complete removal of detergent results in a high molecular weight aggregate.

More than 95 percent of the Gpp(NH)p-binding proteins present in hepatic plasma membranes are not associated with the Gpp(NH)p-activated adenylate cyclase containing fraction. The bulk of the binding proteins appear to be of low molecular weight. Attempts are being made to further purify the low molecular weight form of the Gpp(NH)p binding proteins by affinity chromatography. Studies are also in progress to purify the dispersed adenylate cyclase using adenosine affinity columns in view of the finding that the enzyme contains sites highly specific for the nucleoside. (A. Welton, A. Newby, P. Lad, and H. Yamamura).

D. Structure-Function Relationships in Glucagon

Various chemical modifications of glucagon have been investigated to determine those structural features of the molecule that are necessary for the binding and action of the hormone at its receptor in hepatic plasma membranes. The major findings are summarized as follows:

1. The two tyrosyl residues of glucagon are essential for binding and action.

2. Incorporation of iodine atoms in the tyrosyl residues increases, presumably through increased hydrophobicity, the interaction of the hormone with its receptor.

3. Ionization of the phenoxy groups in tyrosine renders the hormone incapable of reacting with the receptor. Ionization is increased by iodination due to lowering of the pK_a of the phenoxy groups.

Conclusions from points 1-3: Iodinated glucagon is more active than native glucagon at low pH (6.5-7.0) but less active with increasing pH due to decreased concentration of un-ionized tyrosyl residues. Iodinated hormone is a heterogeneous mixture of active (native and iodinated containing un-ionized phenoxy residues) and inactive (iodoglucagon with ionized phenoxy residues) forms of the hormone. Such heterogeneity leads to hazards in employing radio-iodinated glucagon (and possibly other peptide hormones) for evaluating hormone binding at the receptor and probably explains the complex features of the binding and fate of iodinated glucagon when incubated with hepatic membranes. The use of ^{3}H glucagon or of native glucagon (see below) for evaluating the binding process has given much clearer results.

4. Treatment of glucagon with cyanogen bromide gives the derivative [des-Asn^{28},Thr^{29}][homoserine lactone^{27}]glucagon (termed CnBr-glucagon) which displays two percent of the action and binding of native glucagon. It is not known as yet whether the decrease in biological activity is due to the replacement of the methionine residue with homoserine lactone, or by the deletion of the asparagine and threonine residues or both.

5. Semisynthetic analogs of glucagon substituted in the C-terminal region of the hormone were prepared by nucleophilic ring opening reactions at the homoserine lactone C-terminal residue by reaction with various amine nucleophiles. Insertion of hydrazine and aminobutane at the C-terminal end of CnBr-glucagon did not alter the biological activity. However, addition of a bulky substituent such as aminohexyl biotinylamide resulted in marked decrease in binding and action, suggesting that excessive changes in steric and lipophilic properties at the C-terminal end are of more significance than changes in charge. The use of CnBr glucagon for extending the chain length of glucagon may be particularly useful for preparing site directed affinity analogs of glucagon that may be helpful in the isolation of the glucagon receptor.

6. As previously reported, removal of the N-terminal histidine residue converts the hormone into a partial agonist. It has now been found that oxidation of the amino group of the histidine residue surprisingly renders the molecule inactive and incapable of binding to the glucagon receptor.

7. Conversion of the lysine residue at position 12 to homoarginine does not change the biological activity of glucagon. This finding allows selective interactions with the terminal NH_2-group of glucagon and should be helpful in evaluating the effects of substituents added to the N-terminal residue on biological activity. (M. Lin, C. Londos, V. Hruby, S. Nicosia and M. Rodbell).

Binding of Unlabeled and ^{3}H-glucagon to Hepatic Receptors

Because of the problems encountered in studying the binding of ^{125}I-glucagon to the receptors (see above), procedures have been developed for studying the binding of native, unlabeled glucagon to hepatic membranes. The procedure employed is to bioassay - using the highly sensitive adenylate cyclase assay system - glucagon present in the medium before and after incubation with hepatic membranes. The results obtained with this procedure showed that glucagon is rapidly taken up by the hepatic membranes at sites displaying a K_D of about 2 nM; the number of sites is 2- to 3-times that previously evaluated from

^{125}I-glucagon binding. Guanine nucleotides decrease the affinity of the sites for glucagon, as previously observed with ^{125}I-glucagon binding.

Recently, ^{3}H-glucagon has been prepared by catalytic exchange of tritium gas with the iodine atoms of iodinated glucagon. ^{3}H-glucagon is fully active and displays a pH optimum of 7.5 or about one pH unit higher than that of iodinated glucagon. ^{3}H-glucagon binding reaches equilibrium much more rapidly than does ^{125}I-glucagon and agrees with the rapid binding of unlabeled, native glucagon. Studies with ^{3}H-glucagon should provide more definitive evidence on the binding characteristics of glucagon and the kinetic parameters influenced by the actions of guanine nucleotides. Such studies are in progress. (M. Lin and C. Londos).

The Actions of Growth Hormone on Hepatic Cells

A new project has been initiated which concerns the mechanism by which growth hormone affects transcription and translation processes in hepatic cells. Various liver cell lines, in culture, have been investigated for effects of growth hormone on ornithine decarboxylase and ^{14}C-leucine incorporation into proteins. A few show modest, but reproducible effects of the hormone at concentrations of 10^{-10} M, particularly on leucine incorporation. Future studies will evaluate effects on RNA and DNA synthesis. The ultimate goal of the project is to determine whether growth hormone acts through a plasma membrane associated process or though intracellular processes (such as nuclei and ribosomes) such as occurs with several protein toxins (M. Lin).

SECTION ON ENDOCRINOLOGY

Mechanism of Hormonal Regulation of Lipoprotein Lipase Activity in Mammary Gland

Lipoprotein lipase activity is increased in mammary gland and decreased in adipose tissue during lactation. Earlier studies showed in lactating rats that these changes in enzyme activity are mediated through prolactin secretion by the anterior pituitary gland. The present study was initiated to determine the mechanism of hormonal regulation of lipoprotein lipase activity in mammary gland.

Preliminary findings reported last year suggested that factors in addition to prolactin may be needed for stimulating lipoprotein lipase activity in mammary gland of immature rats. Recent studies in pregnant rats showed that lipolytic activity in mammary tissue on the 16th and on the 20th days of gestation was unaffected by prolactin injected for 2-1/2 days. A three-fold increase in enzyme activity of mammary tissue was obtained on the 20th, but not on the 16th, day of gestation when prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was injected for 2-1/2 days. $PGF_{2\alpha}$ also reduced serum triacylglycerol concentration 50% on the 20th but not the 16th day. Analyses of serum showed that $PGF_{2\alpha}$ reduced progesterone concentrations >80% on both days and increased immunoreactive prolactin concentrations 5-fold on the 20th day, but had no effect on prolactin concentrations on the 16th day. These findings suggest that the surge in serum $PGF_{2\alpha}$ prior to parturition on the 21st-22nd day of gestation may be important in the regulation of progesterone and prolactin concentrations in serum, and thereby affect

prolactin-dependent systems. The possibility that lipoprotein lipase activity in mammary tissue may be unresponsive to prolactin in the presence of high concentrations of progesterone in serum is being studied *in vivo* in 20-day pregnant rats and *in vitro* in cultured rat mammary epithelial cells. (P. M. Spooner and R. O. Scow).

Uptake of Triacylglycerol and Cholesterol from Blood by Perfused Lactating Rat Mammary Tissue

Earlier studies showed that perfused mammary tissue of lactating rats takes up and hydrolyzes triacylglycerol from chylomicrons in blood, and that this process is dependent on lipoprotein lipase activity in the capillaries. Lactating mammary tissue also removes triacylglycerol from artificial emulsions but only if they are first incubated with serum. Experiments are in progress to determine the component(s) of serum necessary for uptake of triacylglycerol by perfused mammary tissue. Studies by others showed that hydrolysis of triacylglycerol by lipoprotein lipase *in vitro* requires apolipoprotein C-II, a normal constituent of chylomicrons, very low density lipoproteins and high density lipoproteins (HDL) of serum. Preliminary findings indicate that triacylglycerol in artificial emulsions is taken up and hydrolyzed by perfused mammary tissue if the emulsion is incubated first with human HDL. It is not known yet which component of HDL is involved.

Studies elsewhere have shown that 50-85% of the cholesterol secreted in milk is derived from blood and that cholesterol is readily taken up from chylomicrons by lactating mammary tissue. Recent studies in perfused mammary tissue showed that chylomicron cholesterol is taken up with triacylglycerol and that uptake of both lipids is markedly suppressed when lipoprotein lipase activity is low in the tissue. It is proposed that uptake of triacylglycerol by the tissue requires the action of lipoprotein lipase, whereas uptake of cholesterol is secondary to reduction of the triacylglycerol core, through the action of the enzyme on the core and transfer of the products to tissue and blood. (R. O. Scow, O. Zinder and C. R. Mendelson).

Effect of Prolactin on Lipoprotein Lipase Activity and Uptake of Blood Triacylglycerol in Crop Sac of Pigeons

Prolactin stimulates in pigeons growth of the crop sac and formation of crop "milk", which consists of desquamated epithelial cells containing 12% fat. Last year it was reported that prolactin injections increase markedly lipoprotein lipase activity in crop sac, suggesting that blood triacylglycerol might be a source of fatty acids for the formation of crop "milk". Recent studies showed that uptake of triacylglycerol from both injected chylomicrons and injected artificial emulsions increased with lipoprotein lipase activity in crop sac of pigeons treated with prolactin. Most of the fatty acids retained were present as triacylglycerol, 6% as diacylglycerol, and lesser amounts (>3% each) as monoacylglycerol, free fatty acids and phospholipids. Preliminary radioautographic findings indicate that some of the fatty acids taken up were present, within 10 min, in the cytoplasm and lipid droplets of basal epithelial cells of the crop sac. The results show that blood triacylglycerol is a source of fatty acids for crop sac epithelium, and that lipoprotein lipase

is involved in the uptake of blood triacylglycerol by this tissue (A. A. Ucci, S. S. Chernick and R. O. Scow).

Effects of Lipoprotein Lipase, Phospholipase A_2 and Phospholipase C on Chylomicrons in vitro.

Lipoprotein lipase purified from bovine milk readily hydrolyzed triacylglycerol in chylomicrons stepwise to glycerol and fatty acids if sufficient albumin was present in the medium to bind the fatty acids produced. When lipolysis occurred at maximal rate, however, there was a transient accumulation in chylomicrons of monoacylglycerol, up to 1/3 of that formed, before it was hydrolyzed to glycerol and fatty acids. This delay in hydrolysis probably reflects both positional specificity of lipoprotein lipase for primary ester bonds of acylglycerols and time needed for nonenzymatic isomerization of 2-monoacylglycerol to 1(3)-monoacylglycerol, which can be hydrolyzed by the enzyme to glycerol and fatty acids.

Purified lipoprotein lipase also hydrolyzed phosphatidylcholine of chylomicrons to 2-acyl lysophosphatidylcholine and fatty acid. The rate of hydrolysis which was always less than 5% of that for triacylglycerol, was increased with enzyme concentration and decreased when fatty acid binding sites on albumin were limited in the incubation medium. Others have proposed that hepatic triacylglycerol lipase is principally responsible for phospholipase A_1 activity in postheparin plasma. Our findings indicate that lipoprotein lipase can account for some of the phospholipase activity in postheparin plasma.

Phospholipase A_2 and phospholipase C both hydrolyzed chylomicron phosphatidylcholine, >92% in 10 min, but not triacylglycerol. The resultant phosphatidylcholine-deficient chylomicrons, which could be concentrated by ultracentrifugation and resuspended in incubation medium, were readily depleted of triacylglycerol when incubated with lipase in medium containing sufficient albumin to bind fatty acids formed. Electron microscopic analyses showed that both types of phosphatidylcholine-deficient chylomicrons resembled control chylomicrons in that they were spherical with smooth surfaces.

Water spaces lined by lipolytic products developed in intact chylomicrons when incubated with lipoprotein lipase in medium containing limited albumin. Water spaces also developed, under the same conditions, in chylomicrons pretreated with phospholipase C, but not in those pretreated with phospholipase A_2. Laminar structures were present, however, in the incubation medium of the latter group. Chylomicron remnants, seen as flat sacs by negative staining, were produced when intact chylomicrons or phosphatidylcholine-deficient chylomicrons of either type were incubated with lipoprotein lipase in medium containing excess albumin. These findings indicate that phosphatidylcholine, which accounts for about two-thirds of the lipid in the surface film, can be removed from the film without disrupting the chylomicrons or blocking the action of lipoprotein lipase on triacylglycerol in the core. (R. O. Scow and E. J. Blanchette-Mackie).

A Model for Lipid Transport by Lateral Diffusion in Cell Membranes

In vitro studies reported last year showed that products of lipolysis

(monoacylglycerol and fatty acids) are retained in chylomicrons incubated with purified lipoprotein lipase when fatty acid acceptors are limited in the medium. Morphological studies of these chylomicrons showed that the lipolytic products accumulate in interfacial planes between core triacylglycerol and water, and that the chylomicron surface film is extended as a lipid monolayer lining and spiraling within aqueous spaces that form in the chylomicrons. These results indicated that lipolytic products can move by lateral diffusion in the monolayer, and that lipolysis will continue as long as the monolayer can expand.

Perfusion studies in mammary and adipose tissue showed that uptake of chylomicron triacylglycerol from blood involves hydrolysis of triacylglycerol by lipoprotein lipase to partial glycerides and fatty acids which cross the capillary endothelium to be utilized by the tissue cells. Morphological studies showed that chylomicrons become attached to the capillary endothelium during uptake of triacylglycerol by the tissue, that the luminal surface of the endothelium is connected with the basal surface by membranes lining channels (vesicles and vacuoles) which cross the endothelial cells, and that there are sites of apposition between different cells in tissue. These findings suggested that products of lipolysis within capillaries might move across the endothelium and between cells by lateral diffusion in cell membranes. Therefore the possibility was considered that fusion of the external leaflets of plasma membrane of opposing cells might form a continuous lipid interfacial plane in which lipolytic products could move.

The hypothesis that lipolytic products can locate and move by lateral diffusion in cell membranes is supported by the following observations:

1) Hepatic cells of fed rats contained lipid inclusions which consisted mostly of triacylglycerol with stacks of bilayered lamellae at the periphery. The lamellae, which sometimes extended into the osmiophilic core of the inclusions, were structurally associated also wtih endoplasmic reticulum. The electron-opaque areas within the inclusions were replaced with electron-lucent areas when liver fixed with glucaraldehyde was incubated at 37° C. These findings suggest that the electron-lucent areas result from enzymatic hydrolysis of triacylglycerol with accumulation and diffusion of the amphilic lipid products within the lamellae and endoplasmic reticulum.

2) Mammary tissue of lactating rats contained bilayered lamellae that extended from the endothelial plasmalemma toward the capillary lumen, sometimes in close association with chylomicrons or other lipid particles in blood. Lamellae were also seen in the intercellular space, between capillary endothelium and mammary epithelial cells. In some sections, the intercellular lamellae appeared to be spiraled extensions of plasmalemma of endothelial and parenchymal cells. Lamellae were also present within alveolar cells, spanning the space between the plasmalemma and the bilayered membranes of rough surfaced endoplasmic reticulum, the site of esterification of fatty acids for secretin in milk. Lamellae were sometimes associated with milk lipid droplets near the basal plasma membrane of the epithelial cells.

Prolactin-stimulated crop sac.

Electron microscopic study of the crop sac of pigeons treated with prolactin showed that capillary endothelial cells and basal epithelial cells had

microvilli-like cytoplasmic protrusions which penetrated their respective basement membranes and sometimes apposed each other in the intracellular space. When the crop sacs were fixed with glutaraldehyde at 24° C, the cytoplasmic projections described above often bore lamellar whorls, and lamellae were seen along the lateral borders of basal epithelial cells as well as on the surface of lipid droplets within crop epithelial cells. However, when the tissue was fixed with glutaraldehyde at 0-4° C the number of such lamellae seen was markedly reduced and none were found in tissues fixed first in 2% osmium tetroxide at 0-4° C.

The lamellar structure seen in mammary gland and crop sac probably result from hydrolysis of blood triacylglycerol during and after fixation of tissue with glutaraldehyde, especially at room temperature. It is likely that the amphilic lipids formed, partial glycerides and fatty acids, diffuse in the outer leaflet of the plasmalemma of endothelial cells, along cytoplasmic protrusions, to the outer leaflet at the plasmalemma of parenchymal cells. Also, the amphilic products may accumulate in the outer leaflet of a cell membrane and extend outwardly as bilayered lamellar whorls, since reesterification in these tissues is inhibited by glutaraldehyde. The findings indicate that there is a fusion of the external leaflets of plasmalemma of opposing cells which forms a continuous water-lipid interfacial plane in wich lipolytic products can move by lateral diffusion. (R. O. Scow, E. J. Blanchette-Mackie and A. A. Ucci).

Pharyngeal Lipase

Earlier studies from this laboratory showed that tissues in or near the pharynx in man secrete a lipase that acts in the stomach, at pH 5.4, to hydrolyze triacylglycerol to di-and monoacylglycerols and fatty acids, and that this is the first step in the digestion of dietary fat. A similar activity has been found in fluid collected from the proximal end of the esophageal fistula of an adult patient on the NIAMDD-DD service. About 15 liters of thick fluid were collected over a period of 2 weeks and stored frozen. Mucus in the fluid was liquified by sonication. The lipolytic activity in the fluid is remarkably stable since only 30% was lost when stored for 4 months at 4° C, and none was lost when stored in the frozen state. Purification of the lipase in this fluid is now under way.

Recent collaborative studies at the Department of Pediatrics, University of Oulu, Finland indicates that lipolytic activity is present in the stomach contents of both premature and neonatal infants, and the pH curve of the activity suggests that the activity is due, at least in part, to pharyngeal lipase. This suggestion is supported by the finding of a similar enzyme activity in aspirates from the upper esophagus of a child with esophageal atresia, and in aspirates from the stomach of a child with pyloric stenosis, in which the the stomach. It is of interest that lipolytic activity was found in stomach contents of premature infants with gestational age as young as 33 weeks. Studies are in progress to determine the importance of pharyngeal lipase in digestion in infants. (S. S. Chernick and R. O. Scow).

NUTRITIONAL BIOCHEMISTRY SECTION

Vitamin E Nutrition and Metabolism

A binding protein for α-tocopherol has been found in rat liver supernatant. It has a low molecular weight, is relatively heat stable and appears to be specific for α-tocopherol. The protein was not detectable in other tissues. In studies of the stabilization of the red cell membrane, γ-tocopherol was found to be 38% as active as α-tocopherol in preventing perodixase hemolysis. (J. G. Bieri, G. L. Catignani and S. L. Thorp).

Biochemical Studies of Hepatic and Intestinal Function

Continuing studies of intestinal metabolism have shown that in addition to utilizing glutamine as a primary energy source, glutamate, aspartate and arginine are also extensively metabolized. The intermediates formed were quantitatively determined. In more detailedstudies of the glutamine degrading enzymes in intestine, a highly active phosphate-dependent glutaminase has been localized in mitochondria of both villous and crypt cells. The activity was uniformly distributed along the duodenum, jejunum and ileum. (H.G. Windmueller, L. M.Pinkus and A. E. Spaeth).

VITAMIN METABOLISM SECTION

Studies on Folic Acid

Dihydrofolic reductase which catalyzes the reduction of folic acid or dihydrofolic and to coenzymatically active tetraydrofolic acid continues to be the subject of intensive investigation since it appears to be the primary target enzyme for one of the most efficacious cancer chemotherapeutic drugs, methotrexate. Examination of the highly purified chicken liver enzyme by isoelectric focusing revealed three to four distinct peaks of activity as well as several bands of non-protein impurities. The major peak represents 65 to 80% of the total dihydrofolic reductase activity and exhibits an isoelectric point of 8.4. The enzyme obtained from this isoelectric band is devoid of bound substrates, exhibits a 280/260 absorbency ratio approaching 2 and a specific activity of approximately 14 units per mg of protein.

The molecular parameters of chicken liver dihydrofolic reductase are being examined on the enzyme obtained from the pH 8.4 isoelectric band. The molecular weight has been determined to be 23,000 confirming the unusually low molecular weight for this pyridine nucleotide enzyme. The molar extinction coefficient is 26,690 and the N-terminal amino end is valine. The amino acid composition has been determined. Of particular interest is the observation that it contains a single cysteine residue. This has been confirmed by p-hydroxymercuribenzoate titration in the presence and absence of 6 M guanidine hydrochloride. The interaction of substrates with the enzyme has been measured by determination of the dissociation constants by fluorescence quenching and confirm the tightness of this binding. The circular dichroism spectrum suggests that the enzyme contains very little α-helical structure (B. T. Kaufman and V. Kemerer).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15000-06 LNE

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mechanism of Action of Hormones and Guanine Nucleotides on Adenylate Cyclase Systems.

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

PI: Martin Rodbell Chief, LNE NIAMDD LNE

OTHER: Gordon Hammes, Fogarty Scholar, Fogarty International Center
Professor of Chemistry, Cornell University
Ithaca, New York

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Membrane Regulation Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.20	0.5

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

The actions of guanine nucleotides and glucagon on the adenylate cyclase system in hepatic plasma membranes have been evaluated using an iterative computer fitting process. A relatively simple two state allosteric model was tested and found to quantitatively describe the binding and actions of the nucleotides and the hormone on this system.

PHS-6040
(12-75)

Project Description

We have published previously a model for hormone and guanine nucleotide action on the hepatic adenylate cyclase system in which it was proposed that the enzyme system exists in three fundamental states of enzymic activity, and that these states differ primarily in their affinities for a putative inhibitor, protonated ATP, at the active site. Satisfactory fits were obtained with this model but the complexity of a three state model and the indirect evidence for the putative inhibitory role of protonated ATP compelled us to investigate a simpler model. Moreover, subsequent investigations of this system revealed that the enzyme contains sites for divalent cations (Mn or Mg) which serve to regulate the activity of the enzyme; these findings questioned the role of protonated substrate as a primary regulatory ligand.

A simpler two state model was developed to explain the activation of the enzyme by GTP,Gpp(NH)p, and glucagon and the dependence of the catalytic rate on divalent cations. The basic model proposes that the adenylate cyclase system can exist in two states, A and B; that the activating ligands (guanine nucleotides and glucagon) bind preferentially to the B state; and that only the B state is active. Kinetic data were quantitatively fit to this model from which the binding constants for the interaction of the A and B states with glucagon, GTP, and Gpp(NH)p were obtained. The substrates, ATP and App(NH)p, showed little preference for the A and B states; simple Michaelis-Menton kinetics were sufficient to describe the dependence of the catalytic rate on substrate concentration under optimal conditions. GTP and glucagon displayed 5- and 9-fold, respectively, increases in binding to the B relative to the A state whereas Gpp(NH)p displayed a 100-fold preference. Thus, by mass action, Gpp(NH)p is a far more effective activating ligand than the natural nucleotide, GTP. The allosteric model also provides a simple explanation for the synergistic effects of GTP and glucagon on the enzyme. Subsequent evaluation of the kinetics of Mg and Mn activation with the two state model suggests that the divalent cations react preferentially with the active B state of the enzyme system. Thus, the two state model provides a simple conceptual framework for evaluating the kinetic characteristics of the enzyme in the presence of activating (or inhibitor) ligands.

The complex nature of adenylate cyclase systems and the often confusing and contradictory data obtained in numerous laboratories necessitates the formulation of a conceptual framework that can help to resolve some of the apparent complexities of the problem. To the extent that the proposed two state allosteric model provides a conceptual framework that simplifies our notions of hormone action, the present work is a contribution to research in the field of hormone action, one of the most important areas of biomedical research.

Further evaluation of the two state allosteric model requires identification of the macromolecular components of the adenylate cyclase system and development of means for directly assessing the interaction of the various ligands (hormones, guanine nucleotides, and metal ions) with the components. Such efforts are in progress.

Publications

Hammes, G. and Rodbell, M.: A Simple Model for Hormone Activated Adenylate Cyclase Systems. Proc. Natl. Acad. Sci. U.S.A. 73: 1189-1192, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15001-02 LNE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structure-Function Relationship for Glucagon

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

PI:	Michael C. Lin	Research Chemist	LNE NIAMDD
OTHER:	Simonetta Nicosia	Visiting Fellow	LNE NIAMDD

COOPERATING UNITS (If any)

None

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Membrane Regulation Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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

The goal of this project is to determine the structural requirement of glucagon for binding to its receptor and for action on the adenylate cyclase system. The study on iodination of tyrosyl residues showed that increased hydrophobicity at Tyr-10 and/or Tyr-13 enhances the potency of glucagon while ionization of tyrosyl residue has deleterious effect on the binding and activity of glucagon. Tritium labeled glucagon has now been prepared and extensively purified. Preliminary studies indicated that the binding of ^{3}H-glucagon differs from that of ^{125}I-glucagon in several aspects.

Project Description:

Objectives: The goal of this project is to determine the structural requirement of glucagon for binding and action at its receptor. New understanding can be gained by studying the effect of chemical modification on the hormone.

Methods: Iodoglucagon was prepared by choramine T procedure followed by purification with column chromatography. ^{3}H-glucagon was preared by catalytic reduction of iodoglucagon with tritium gas and followed by extensive purification. The biological activity of glucagon derivatives was determined by their ability to activate the adenylate cyclase system in liver. The binding was measured by their interaction with the receptor site in the hepatic plasma membrane.

Major Findings: Iodoglucagon, at pH 7.0 and below, binds to the receptor and activates adenylate cyclase with an affinity about 3-fold higher that that of native glucagon. At pH 8.5, the affinity of the native glucagon for its receptor is the same as that seen at pH 7.0. However, iodoglucagon binds with a lowered affinity with increasing pH. Therefore, we conclude that (i) incorporation of iodine atoms in the tyrosyl residues increases hydrophobic interaction of the hormone with its receptor and (ii) ionization of the phenoxy group results in the loss of biological activity. Thus, the tyrosyl residues in glucagon are critically involved in the function of the hormone. In view of the major effect due to iodination of the hormone, tritium labeled glucagon has been prepared for the studying of its binding to the receptor. Preliminary experiments indicate that the binding of ^{3}H-glucagon differ from that of ^{125}I-glucagon in several aspects. The major differences were observed in the optimal pH and the rate of the binding for these two radioactive glucagons to the receptor.

Significance to Biomedical Research and the Program of the Institute: The knowledge of the structural requirement of heromone for its function is fundamental to the understanding of hormone action. Glucagon derivatives, prepared or currently under preparation, may have potential diagnostic or therapeutic value.

Proposed Course of Project: The interaction of glucagon with its receptor will be examined extensively through the use of ^{3}H-glucagon. Chemical modification of the hormone will be continued. Major effort will be directed toward the alteration of NH_2-terminal region which seems to be essential in the activity of glucagon.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15QQ2-02 LNE

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Binding of Unlabeled Glucagon to Rat Liver Membranes.

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

PI: C. Londos Staff Fellow LNE NIAMDD

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Nutrition & Endocrinology

SECTION

Membrane Regulation Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.3	0

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

Termination of project.

PHS-6040
(12-75)

Project Description

Objectives: The method for determining binding of native glucagon to liver membranes and the fact that there are differences between native and iodinated glucagon were outlined in the previous project report. Further studies have revealed that binding of native glucagon to rat liver membranes exhibits a K_d of approximaely 1 nanomolar, and that there are 7-10 picomoles of glucagon binding sites per mg membranes protein. The higher affinity for native, as opposed to iodinated glucagon, and the greater total binding of native hormone account for the rapid loss of glucagon activity when combined with liver membranes. Thus, the process previously identified as specific inactivation of glucagon can be accounted for by binding of hormone to membrane receptor sites.

Proposed Course of Project: The project is now being written for publication and will be terminated subsequently.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15003-02 LNE

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Analysis of Adrenal Adenylate Cyclase.

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

PI: C. Londos Staff Fellow LNE NIAMDD

COOPERATING UNITS (If any)
NONE

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Membrane Regulation Section

INSTITUTE AND LOCATION
NIAMDD NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

Project being terminated.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15004-01

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mechanism of Action for Growth Hormone

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

PI: Michael C. Lin Research Chemist LNE NIAMDD
OTHER: None

COOPERATING UNITS (If any)

None

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Membrane Regulation Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

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

The objective of this project is to determine the mode of action for growth hormone. Conditions for cell culture responsive to growth hormone have been developed. A cell line, originally derived from rat liver, has been shown to increase its rate of leucine incorporation after exposure to the hormone. With the use of this cell culture as an assay system, initial experimental approach will be based on a working hypothesis that growth hormone shares common pathway with some of the toxins.

PHS-6040
(12-75)

Project Description:

Objective: The goal of this project is to elucidate the mechanism of action of growth hormone and to determine the role of the cell membrane in the expression of hormone action. There is one class of hormones namely growth hormone, insulin and a few others, in which the action is not mediated directly through cyclic AMP and is poorly understood. There is a possibility that this type of hormone exerts the primary effect directly at the level of transcription or translation. If this were the case, then the cell membrane should be capable of transporting the hormone or fragment of it into the cell. An example of this kind of pathway is seen in the mode of action of toxins such as diptheria toxin, abrin and ricin. The initial approach to this project has been based on this kind of working hypothesis.

Method: Several lines of cell culture, derived originally from rat liver, have been maintained for the purpose of establishing an assay system for growth hormone. Binding of ^{125}I-growth hormone has been used to select the cell lines potentially responsive to this hormone. Overall protein biosynthesis, in terms of radioactive leucine incorporation, is currently used to detect response of the cells to growth hormone. Other types of activity such as DNA and RNA synthesis, membrane transport and ornithine decarboxylase activity, will also be measured as a possible response to the hormone. Various types of toxins will be used as tools to determine whether there is any overlap between pathways for toxin and the hormone. Attempt will be made to localize the hormone or its fragment after the exposure of the cells to growth hormone.

Major Findings: Two cell lines have been shown to bind growth hormone and be responsive to it in terms of enhanced rate of ^{14}C-leucine incorporation. The response of cell culture seemed to be more easily detectable if the culture became confluent before the addition of growth hormone. The presence of serum during the exposure to the hormone appeared essential in obtaining increased rate of leucine incorporation. This type of response is very sensitive; it reaches saturation at 10^{-9} M of bovine growth hormone.

Significance to Biomedical Research and the Program of the Institute: The knowledge of the detailed mechanism for growth hormone action is essential to the full understanding of regulation of life process. The availability of a sensitive assay system for growth hormone has broad application not only in basic research but also has potential use in diagnosis of some diseased states.

Proposed Course of Project: The assay system for growth hormone still requires improvement. The enhanced rate of leucine incorporation due to growth hormone is only 150% of the control value; currently, efforts are being made to increase this response by optimizing various factors involved in the assay. Growth hormone, labeled with radioisotope at several positions, will be propared in order to follow the location and fate of the hormone after its interaction with the cells. The role of cell membrane in the recognition and/or expression of hormone action will be examined in depth.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 15005-01 LNE

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Hepatic Adenylate Cyclase

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

PI: C. Londos Staff Fellow LNE NIAMDD

COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Membrane Regulation Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.5	.5

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

The actions and interactions of ligands that affect the rat hepatic glucagon-sensitive adenylate cyclase system have been investigated. The divalent cations, Mg^{2+} and Mn^{2+}, activate the enzyme system by a mechanism independent of the concentration of uncomplexed ATP in the medium. This rules out models in which the effects of divalent cations are attributed to a lowering of the free, uncomplexed ATP concentration. Two activators of the enzyme system, glucagon and guanine nucleotides, appear to increase the affinity of the system for the cations. Adenosine is a specific inhibitor of the system, and the activating ligands, particularly divalent cations and glucagon, increase the affinity for the nucleoside. Knowledge of the interrelationships of these ligands permits interpretation of kinetic data on adenylate cyclase.

PHS-6040
(12-75)

Project Description

Objectives; Adenylate cyclase systems in eukaryotic cells play an important if not essential role in the expression of hormone action. In addition to hormones, numerous ligands, including substrate, guanine nucleotides, and metal ions, have marked effects on enzyme activity. The multiple ligand interactions have led to a confusing and often controversial literature on the properties of the enzyme. It is the purpose of these studies to sort out, in a systematic fashion, the effects of the various ligands in order to better understand the regulatory process.

Methods: Rat liver plasma membranes were isolated and adenylate cyclase was assayed by standard procedure.

Major Findings: We have strong evidence indicating that the stimulatory effects of divalent cations results from a direct action of the Me^{2+} on the rat liver adenylate cyclase system. For example, low concentrations of Mn^{2+} (0.2 mM) nearly triples the activity seen in the presence of a relatively high concentration of Mg^{2+} (5 mM); such effects are independent of the level of uncomplexed ATP in the assay medium. These results argue for a direct action of cations on the system, and seem to rule out models in which the activation by Me^{2+} is attributed to a lowering of the concentration of uncomplexed ATP, which is thought to be a potent inhibitory of adenylate cyclase activity. The affinity of the metal ion-binding site for Mn^{2+} is at least 10-fold greater than that for Mg, and Ca^{2+} appears to inhibit activity by binding to this site. Adenosine is a specific inhibitor of the liver adenylate cyclase system, and the apparent K_i is strongly dependent upon the state of activation of the enzyme. For example, saturation of the Me^{2+}-binding site or the presence of glucagon leads to a nearly 20-fold increase in the affinity for the inhibitory nucleoside. Since adenosine is present in most adenylate cyclase assay media, either as an contaminant or metabolite of ATP or cyclic AMP, and since activating ligands increase the affinity for adenosine at the inhibitory site, the kinetics of the system were understandably confusing. If care is taken to eliminate the nucleoside by reducing the concentrations of its source or by the addition of adenosine deaminase, kinetic data become more easily interpretable. For example, we can now show that both guanine nucleotides and glucagon seem to increase considerably the affinity of the Me^{2+}-binding site for $Mg2+$.

Further studies with numerous adenosine analogs have revealed a high degree of specificity, particularly on the purine ring, where even minor substitutions or alterations in structure results in a loss the inhibitory effect. Minor changes in the ribose portion are tolerated, and some deoxyribose analogs are even more potent than adenosine.

The above studies have identified two regulatory sites in the glucagon-sensitive adenylate cyclase system from rat liver, one for metal ions and another for adenosine. The glucagon receptor and the guanine nucleotide site are linked somehow to these sites, and all four sites are linked structurally, since a change in one ligand affects the interaction of the ligands at the three remaining sites.

Significance to Bio-Medical Research and the Program of the Institute. The glucgon-adenylate cyclase system is an important regulator of carbohydrate metabolism, and it has been suggested that elevated blood glucagon contributes to the hyperglycemia of diabetes. Thus, to understand the regulation of adenylate cyclase is obviously important.

Adenosine as a metabolic product of cyclic AMP, may serve as a feedback inhibitor of adenylate cyclase. Moreover, glucagon, the very signal that stimulates the enzyme system, also sensitizes the system to inhibition by adenosine.

Proposed Course of Project: We propose to continue to define the relationships between the effects of ligands which interact with the hepatic adenylate cyclase system.

Publications:

Londos, C. and Rodbell, M.: Adenylate Cyclase: Actions and Interactions of Regulatory Ligands. In Roberts, G. C. K. (Ed.): Drug Action at the Molecular Level. London, Macmillan Press (in press).

Rodbell, M. and Londos, C.: Regulation of Hepatic Adenylate Cyclase by Glucagon, GTP, Divalent Cations, and Adenosine. Metabolism (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15006-01 LNE

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Purification of Hepatic Adenylate Cyclase

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

PI:	Martin Rodbell	Chief, Laboratory	LNE	NIAMDD
OTHER:	Pramod Lad	Visiting Fellow	LNE	NIAMDD
	Simonetta Nicosia	Visiting Fellow	LNE	NIAMDD
	Andrew Newby	Guest Worker	LNE	NIAMDD
	Ann Welton	Postdoctoral Fellow	LNE	NIAMDD

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Nutrition & Endocrinology

SECTION
Membrane Regulation Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

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

Adenylate cyclase in rat liver plasma membranes has been solubilized with the use of detergents. Procedures have been developed for assaying the binding of radioactive Gpp(NH)p and glucagon to various fractions obtained after detergent solubilization of membranes and subsequent chromatography. It is concluded that most of the guanine nucleotide binding sites are not associated with purified adenylate cyclase; the purified enzyme is activated by Gpp(NH)p indicating that the guanine nucleotide regulatory component remains associated with adenylate cyclase during purification. The purified enzyme is activated by glucagon. However, a crude solubilized fraction can be restored partially to a glucagon-sensitive state after removal of detergents by dialysis. These findings indicate that coupling of the glucagon receptor to the detergent dispersed adenylate cyclase system can be re-constituted. The role of membrane lipids in the reconstitution process is being investigated.

PHS-6040

Project Description

Objectives; To solubilize, purify, and characterize the catalytic component, glucagon receptor, and guanyl nucleotide binding protein of the glucagon-sensitive adenylate cyclase system present in rat liver plasma membranes and to use these components to reconstitute a soluble, hormone-sensitive adenylate cyclase system.

Methods Employed: The catalytic and guanyl nucleotide binding components of the adenylate cyclase system were solubilized from purified rat liver plasma membranes using the non-ionic detergent, Lubrol PX. Solubilization was carried out in the presence of high concentrations of sucrose to stabilize adenylate cyclase activity. Adenylate cyclase was assayed by conventional techniques. Guanyl nucleotide (^{3}H-Gpp(NH)p and ^{3}H-GTP)-binding was carried out using a forced dialysis technique.

Partial purification of the catalytic and guanyl nucelotide-binding components was carried out by molecular exclusion chromatography on Sepharose 6B or Ultragel AcA 22 (both gels having exclusion limits of approximately, 1,000,000) in the presence of low concentrations of Lubrol. The separation of these cyclase components from other membrane enzymes was assessed by assaying the column for 5'-nucleotidase, ATPase, protein, and phospholipid using conventional assay techniques. Further purification of the cyclase catalytic component was attempted using chromtography on DEAE-, CM-, and hydrophobic-agarose resins. Purification was also attempted using polyacrylamide gel electrophoresis in the presence of detergent, pH precipitation, and ammonium sulfate fractionation.

Lubrol PX and sodium cholate were used to solubilize the glucagon receptor from purified rat liver membranes. The solubilization was carried out on membranes which had been pretreated with ^{125}I-glucagon under conditions where the hormone is reversibly bound to the membranes. The "tagged" receptor was separated from free glucagon by chromatography on Agarose 0.5 M at pH 6.5. This "tagged" receptor was then used for developing a rapid method for the assay of glucagon binding to solubilized receptor and to characterize the solubilized receptor with regard to the effect of guanyl nucleotides, pH, and dilution on hormone dissociation.

Major Findings: Of a wide variety of detergents studied for their ability to solubilize adenylate cyclase, Lubrol PX was chosen since it caused the least inactivation of the enzyme during solubilization. Two forms of adenylate cyclase were studied in these investigations; one being a highly activated form of the enzyme produced by pretreating membranes with glucagon and Gpp(NH)p (henceforth referred to as pretreated enzyme) and the second being enzyme from untreated membranes (subsequently referred to as untreated enzyme). Lubrol solubilized nearly 100% of the pretreated enzyme from membranes but the untreated enzyme was much more sensitive to detergent treatment in that only 30% of its activity could be recovered after solubilization. Even though the pretreated enzyme appeared to be stable during the initial solubilization step, this form of the enzyme too was labile during subsequent purification. Use of high concentrations of sucrose improved stability during purification however.

Chromatography of the pretreated solubilized cyclase on either Sepharose 6B or Ultragel AcA 22 in the presence of a low concentration of lubrol partially purifies the cyclase from the bulk of the lubrol and other solubilized membrane components and results in a 3 to 5 fold increase in the specific activity of the enzyme. Under such chromatography conditions, the cyclase activity is included in the column, migrating as a protein of approximately 350,000 daltons. The other plasma membrane-associated enzymes, 5'-nucleotidase and ATPase are eluted in the void volume of the column. The bulk of the solubilized proteins and phospholipids migrate in two peaks one of which also elutes in the void volume of the column, the second eluting after the cyclase activity. A comparison of the SDS-polyacrylamide gel electrophoresis profiles of the column cyclase fractions and plasma membranes also indicate that many of the protein components of plasma membranes have been separated from adenylate cyclase. The protein profile of the cyclase fractions is still complex, however, indicating the presence of at least 15 polypeptide chains. After chromatography, the partially purified adenylate cyclase also has associated phospholipid in approximately the same phospholipid to protein ratio as is found in the plasma membrane. The major phospholipid associated with the enzyme is phosphatidyl choline.

The migration characteristic of cyclase on molecular sieving gels are dependent upon the inclusion of detergent in the elution buffer. In the absence of detergent, the cyclase forms a high molecular weight aggregate and elutes in the void volume of the column. Under such conditions then there is little purification of the enzyme from other membrane components.

Attempts at further purifying pretreated column chromatographed cyclase have been unsuccessful. Purification methods such as polyacrylamide gel electrophoresis, isoelectric focusing, and such as DEAE-, CM-, and hydrophobic-agarose chromatography do not inactivate the enzyme but will not separate the cyclase from other contaminating proteins either because all the proteins have very similar size and charge properties or are associated together in the form of a detergent phospholipid, and protein aggregate. The observation that the migration characteristics of cyclase on molecular sieving gels are dependent upon the presence of detergent in the elution buffer and the possibility that lubrol solubilization results in the association of cyclase with a detergent phospholipid, and protein aggregate casts doubts on the validity of any previous measurements of the molecular weights of solubilized forms of adenylate cyclase which have been made without purifying the enzyme.

Within these limitations it has been observed that there is no detectable difference in Stokes radius between pretreated and untreated solubilized adenylate cyclases. In the latter case it was possible to show that the catalytic unit and the Gpp(NH)p binding protein were still functionally associated since the activity was still stimulated by Gpp(NH)p. These observations argue against a mechanism of nucleotide activation which would require the binding of the nucleotide followed by dissociation of the binding protein from the catalytic component for activation of the cyclase system. Also of interest in this regard is the observation that the majority of the guanyl nucleotide-binding protein solubilized from plasma membranes is separated from adenylate cyclase during column chromatography and appears to have a lower molecular weight than the

cyclase. This suggests that the majority of the binding protein present in the membrane is either not associated with the adenylate cyclase system or that spare binding proteins are present in the membrane.

Studies of the solubilized glucagon receptor were carried out using a hormone-receptor complex which was obtained from rat liver plasma membranes pretreated with ^{125}I-glucagon. Since detergent interferes with the binding of glucagon to its receptor, sodium cholate was used in preference to lubrol for these studies because cholate can be much more easily removed from proteins after solubilization. After cholate treatment, the hormone-receptor complex was separated from both free hormone and detergent by gel chromatography and used in attempts to develop a rapid assay for the binding of glucagon. A variety of techniques were used to determine whether differences in size, charge or both of the receptor and hormone could be used to effect their separation in a binding assay. These efforts have been unsuccessful but attempts to develop a filter with appropriate charge and size properties to allow the passage of glucagon but not solubilized receptor are still in progress. Until such a method is developed we are using the slower, more tedious Hummel-Dreyer procedure.

The "tagged" receptor was also used to study the physical properties of the complex after solubilization. The following results were obtained: 1) GTP dissociates the hormone from the solubilized receptor. The rate of dissociation is similar to that seen in the intact membrane, though the kinetics of dissociation are complex, 2) alkaline pH's lower the dissociation rate, 3) a rapid dissociation of the complex occurs upon dilution, and 4) ionic strength does not effect the rate of dissociation.

In addition, attempts were made to find conditions using sodium cholate which could be used to solubilize both the hormone receptor and an active form of adenylate cyclase to investigate their interrelationships. Since pretreatment of membranes with Gpp(NH)p or hormone and Gpp(NH)p is known to produce states of the enzyme which are more stable to solubilization, it was of interest to study the effect of various activity states induced by Gpp(NH)p on the binding of glucagon to plasma membranes and on the properties of the solubilized hormone-receptor complex. The results showed that treatment of membranes with Gpp(NH)p prior to treatment with ^{125}I-glucagon left the level of binding, the yield in solubilization, and the properties of the soluble receptor unchanged. One wonders if GTP, known to dissociate ^{125}I-glucagon from untreated cyclase, will also induce the dissociation of the hormone from pretreated cyclase. Also it appears that GTP is more potent than Gpp(NH)p in reducing the levels of bound hormone, while the opposite is true for the ability of the nucleotides to activated cyclase. These results suggest the possibility of GTP sites involved in the down-regulation of hormone levels which are separate from those involved in the activation of adenylate cyclase. The relation of "hormonal" and "cyclase" type-nucleotide sites will be investigated both in membranes and in solubilized receptor and cyclase preparation.

Significance to Bio-Medical Research and the Program of the Institute: The important role of adenylate cyclase in hormonal control of cellular growth and metabolism is well documented. To date, however, much of our

knowledge of this complicated regulatory system has been descriptive in nature suggesting a complex role for several ligands (hormone, guanyl nucleotides, adenosine, and metal ion) in the regulation of this enzyme. Through studies using purified components of this enzyme complex it should be possible to more fully understand the mechanism of regulation by these ligands and therefore how this enzyme participates in the control of cellular functions.

Proposed Course: In future studies attempts will be made to further purify solubilized column chromatographed adenylate cyclase using affinity chromatography on resins containing various ligands known to specifically interact with adenylate cyclase. Of particular interest for this approach is the observation from this laboratory that Mn^{+2} and adenosine interact synergistically with cyclase. Therefore affinity chromatography using an adenosine ligand may be especially useful.

Studies will also be made of the characteristics of the guanyl nucleotide-binding protein associated with partially purified adenylate cyclase. The properties of this binding protein will be compared to those of the bulk of the nucleotide binding protein which is separated from cyclase by column chromatography. On the basis of such studies it should be possible to determine if the non-associated binding proteins are different from the associated or might actually be "spare" binding proteins. Also using affinity columns prepared from guanyl nucleotide derivatives, attempts will be made to separate the binding protein from adenylate cyclase, to determine if such dissociation results in the loss of nucleotide regulation of cyclase activity, and to determine if conditions can be established for reconstitution of nucleotide regulation. In this regard it will be interesting to see if recombination can also occur with the "bulk" nucleotide binding protein which separates from cyclase on molecular sieving columns. If a nucleotide affinity column can be obtained which will separate the binding protein from the cyclase, this should also be useful in purifying the binding protein.

Attempts will also be made to determine the migration properties of the glucagon receptor in relation to cyclase and the nucleotide-binding protein on molecular sieving columns. This hopefully will lead to some knowedge of the interrelationships of the catalytic, nucleotide-binding, and receptor components of the cyclase system. Also, using glucagon affinity columns attempts will be made at further purifying the hormone receptor.

Finally, if purified forms (or even partially purified forms) of the catalytic unit, receptor, and nucleotide-binding, protein of the cyclase complex can be obtained, studies will be conducted to reconstitute the hormone-sensitive system from the components. Due to previous investigations suggesting a role for lipids in the activation process, it is very likely that such reconstitution studies will also require the addition of specific phospholipid components back to the system. At present preliminary studies are underway to investigate the role of phospholipids in the reconstitution of hormone sensitivity to systems which contain solubilized catalytic, receptor, and nucleotide-binding proteins.

Publications:

Yamamura, H. and Rodbell, M.: Hydroxybenylpindolol and Hydroxybenzylpropranolol, Partial Beta Adrenergic Agonists on Adenylate Cyclase in the Rat Liver Adipocyte. J. Mol. Pharm. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15100-06 LNE

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Protein-Nucleic Acid Interactions: Chromatin Structure.

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

PI:	Robert T. Simpson	Surgeon (R) (P)	LNE NIAMDD
OTHER:	James P. Whitlock, Jr.	Staff Fellow	LNE NIAMDD
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COOPERATING UNITS (If any)

NONE

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	1.5

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

Hela cell nucleosomes have been prepared as a highly homogeneous population containing 140 ± 5 base pairs of DNA. During the digestion of nuclear DNA to produce nucleosomes, a 60-70 base pair bridging DNA region between nucleosomes is destroyed. Histone H1 appears to bind to the bridging DNA. The nuclesomes contain 1.2 gm histone/gm DNA. Histone H2B reacts with specific anti-H2B antibody while complexed in the nucleosome. Immunosedimentation demonstrates that all nucleosomes contain identical complements of histones, two each of H2A, H2B, H3 and H4. Physicochemical characterization of DNA structure within the nucleosome has been carried out. The role of histone-histone interactions in stabilization of DNA conformation in nucleosomes has been evaluated by solvent perturbation, thermal denaturation and protein cross-linking experiments. The DNA of intact nucleosomes can be labeled at the 5' termini with ATP and polynucleotide kinase. Using labeled particles, we show that there is a potential single strand nick site for DNase I every ten nucleotides along the DNA within nucleosomes - these sites vary widely in their actual susceptibility to the nuclease. Chemical modification studies of histones in chromatin are consistent with models for nucleosome structure which envision hydrophobic interactions between the C-terminal portions of the histones and electrostatic DNA-amino terminal binding.

PHS-6040

Project Description:

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

Methods Employed: Nuclei are prepared from chicken erythrocytes, rodent liver, or Hela cells, labeled with ^{3}H-thymidine or unlabeled. Nuclear suspensions in isotonic sucrose containing 0.1 - 1 mM $CaCl_2$ are digested for varying lengths of time with micrococcal nuclease and nucleosomes isolated by sedimentation on isokinetic sucrose gradients.

Histones are isolated from calf thymus and fractionated into five purified species. Antibodies are elicited to the purified histones in rabbits. To purify antibody, purified histone is coupled to Sepharose after activation with cyanogen bromide. Antisera is adsorbed to the affinity adsorbent and non-bound proteins eluted with a phosphate buffer containing 0.5 M NaCl and 0.5% Triton X-100, a nonionic detergent. Purified antibody is eluted by dissociation of the complex with 0.5 M ammonia, pH 11.8. Antibody reactions with antigens are measured by the micro complement fixation technique.

Whole nuclei or isolated nucleosomes are digested for varying lengths of time by the endogenous endonuclease of rodent liver, micrococcal nuclease, or DNase I. DNA is isolated using a sodium dodecyl sulfate - phenol procedure, precipitated with ethanol and dried. The DNA samples are dissolved in a sample buffer and analyzed by electrophoresis on one of several different polyacrylamide gels, without or with heating to 100° for five minutes to measure double or single strand chain lengths respectively. Gels are stained with Stains-All, scanned and photographed. When samples labeled with ^{32}P are analyzed, radioautography is carried out using No-Screen X-ray film. Standard DNA fragments for size calibration of the electrophoretic gels are prepared by digestion of SV-40 DNA with Hae-III restriction endonuclease.

Standard physical methods are utilized to investigate structure of nucleosomes; including analytical ultracentrifugation on self-generating D_2O density gradients, thermal denaturation, viscometry, and circular dichroism spectrophotometry.

Chemical modification of chromatin or isolated nucleosomes is carried out with dimethylsuberimidate or Lomant's reagent to cross-link the histones, or with ethyl acetimidate or acetic anhydride to yield monofunctional modifications of lysyl residues. Size distribution of the cross-linked histones is made by gel electrophoresis on 5% polyacrylamide gels. Cross-links induced by Lomant's reagent are reversed by heating with β-mercaptoethanol and the histones identified by electrophoresis on 18% polyacrylamide gels.

Histones extracted from chromatin modified with ^{14}C-acetic anhydride are further modified with cold acetic anhydride. Tryptic digestions are carried out and the digests fractionated by gel permeation chromatography and two

dimensional paper or thin layer fingerprints. Radioactive peptides are localized by autoradiography, eluted and repurified if necessary. The amino acid composition of the peptides is determined using a modified JEOL amino acid analyzer employing microbore columns and o-phthaldehyde as detection reagent. Amino terminal groups are determined by a micro-Edman procedure.

The 5' terminus of the DNA of isolated nucleosomes is labeled by transfer of the γ-^{32}P from ATP to nucleosomes by polynucleotide kinase. Modified nucleosomes are separated from enzyme and ATP by sucrose gradient centrifugation. Modified nucleosomes are mixed with nucleosomes labeled with ^{3}H-thymidine to determine the kinetics of DNA digestion for termini and bulk DNA.

Major Findings: Nucleosomes were incubated with purified antibodies to histones H2A, H2B and to hemoglobin and the resulting complexes analyzed by ultracentrifugation. Of these, only anti-H2B bound specifically to nucleosomes. When sufficient antibody is present, essentially all the nucleosomes sedimented with increased velocities suggesting that all chromosomal particles contain H2B. The amount of antibody reacting with H2B in the nucleosome was quantitated by densitometric scanning of gel electrophoresis patterns of the proteins in various nucleosome-anti-H2B complexes separated by sedimentation on sucrose gradients. Under conditions where all particles had increased sedimentation velocities, from 1 to 3 IgG molecules are bound to each nucleosome, the ratio increasing from top to bottom of the sedimenting peak. When nucleosomes are dispersed on the basis of reaction with anti-H2B, the quantitative ratios among the four small histones are identical for all fractions. It thus seems likely that each nucleosome has a similar histone complement, two molecules each of H2A, H2B, H3, and H4. The variation in amount of bound antibody to nucleosome probably reflects a normal distribution during the titration, although differential exposure of H2B antigenic determinants in several populations of nucleosomes can not be excluded as an explanation.

Digestion of rat liver nuclei by an endogenous endonuclease generates double-stranded DNA fragments which are initially about 205 base pairs long, as previously reported by Hewish and Burgoyne. As digestion proceeds, the average size of these fragments is reduced to 150-160 base pairs. Digestion of Hela nuclei with micrococcal nuclease leads to analogous results, intitial production of 200 base pair fragments or multiples therof with later nibbling of these particles to form a relatively stable 140 base pair fragment. The double strand DNA fragments from the endogenous endonuclease digestion when analyzed under denaturing conditions, are seen to contain single strand nicks at ten base intervals. Fifteen bands, 10-150 bases, are clearly resolvable. DNA fragments of 160-200 nucleotides are not resolved as distinct species. These data suggest that the nucleosome or chromosomal subunit contains both a 160 base pair DNA segment, in a conformation susceptible to nicking at ten base intervals, and a forty base pair DNA segment in a conformation more uniformly susceptible to nuclease digestion, that is the "bead and bridge" model derived from morphological observations of Olins and Olins.

Digestion of Hela nuclei gives this same result when the nuclease treatment is performed in 0.15 M NaCl, suggesting that the bead and bridge conformation of

chromosomal DNA exists at physiologic ionic strengths. Removal of some non-histone chromosomal proteins and all of histone H1 by extraction of nuclease prepared chromatin with 0.6 M NaCl increases the rate of digestion to form acid soluble material, reduces the amount of DNA which appears as multiples of the unit length of 200 base pairs, and makes much more rapid the digestion of the 40-50 base pair bridge DNA segment. Thus, H1 appears to either bind to or in some other fashion affect only the digestion properties of the bridge DNA.

With this knowledge of the effects of salt concentration and presence or absence of H1 on digestion progress, it has been possible to prepare highly homogeneous monomeric nucleosomes from HeLa cells. Nuclei are briefly digested to prepare a high molecular weight chromatin. This chromatin is then digested in 0.15 M NaCl with micrococcal nuclease and the digest fractionated by sedimentation on isokinetic sucrose gradients containing 0.1 M NaCl. The product monomeric nucleosome has a histone to DNA ratio of 1.2 gm/gm, contains equal ratios of the four smaller histones and has a single unnicked piece of DNA 140 ± 5 base pairs in length. The melting profile of the nucleosome is like chromatin although the total hyperchromicity is greater. These particles sediment as a homogeneous zone with a sedimentation coefficient of $11.0 \times 10^{-13} sec^{-1}$. The circular dichroism spectrum of nucleosomes has a positive maximum at 280 nm with ellipticity of 1900°, compared with ellipticities for chromatin of 4500° and DNA of 10000°. Urea, 6 M, alters the circular dichroism spectrum to be essentially identical with that of DNA, generates three melting components, and decreases the sedimentation coefficient to 6 S. DNase I and micrococcal nuclease further digest the nucleosome DNA to yield digest fragments characteristic of their digestion of nuclear DNA. The nucleosome thus represents a component of, but not all of, the DNA in the nucleus. This isolation of a pure population of chromatin beads, trimmed of bridge DNA, should facilitate further analyses of the structure of the histone-DNA complex.

Folding a 200 base pair length of DNA around a core of eight histones to form a nucleosome might be expected to be a critical feature in generation of chromatin structure. We approach the thermodynamics and mechanism of folding DNA around histones by both disassenbly of nucleosomes and reconstitution of chromatin particles. Monomeric nucleosomes from chicken erythrocytes appear to melt in an all-or-none transition. Histone-histone dissociation accompanies strand separation since octameric molecules seen by cross-linking nucleosomes at room temperature are not found in melted nucleosomes. In contrast, the melting of higher order nucleosome structures is more complex with histone hexamers and tetramers being detected by cross-linking at several stages in the disassembly of the nucleoprotein. Reconstitution of nucleosomes has thus far been carried out simply by dissociation in 2.5 M NaCl or salt-urea mixtures followed by annealing in 0.6 M NaCl, with or without 1.0 M urea. Fidelity of reconstitution as assessed by circular dichroism spectroscopyis about 95% and the time course for the association is rapid (less than 30 sec for 85% completion). Urea has little effect. As an additional criterion of correct reassociation, nucleosomes were digestd with DNase I and the pattern of single-strand fragments produced compared for whole and reassociated particles. Here the time course of association is slower although the eventual product is again closely similar to the original particle. DNA may fold rapidly around a histone

core and then more slowly slide into the position of optimal interaction with the basic groups of the proteins.

For some reconstitution experiments we wished to have histone cores cross-linked. Dimethylsuberimidate was chosen as a cross-linking agent since its modification product with lysyl amino groups still has a positive charge. Further, chromatin or nucleosomes were modified with ethyl acetimidate to provide a control for the direct effect of the modification on physical properties - amidination providing the same substituent on lysine but lacking the cross-link. In most properties the two modified forms of chromatin and the native resemble one another closely. In the case of melting behavior, a rather large stabilization of the cross-linked modified nucleosomes relative to amidinated or unmodified material was demonstrable. Histone-histone dissociation must be a prerequisite for strand separation in disassembly of nucleosomes by thermal denaturation.

A number of histone peptides modified in chromatin by acetic anhydride have been isolated in pure form and placed in the known primary sequence of the four smaller histones. While currently incomplete, several clear generalizations derive from specific radioactivities of the modified lysyl residues. The amino terminal regions of all four histones have high and constant levels of modification suggesting their ready availability on the surface of the multimeric histone. Data for the central regions of the molecules are sparse due to large insoluble core peptides but for two lysyl residues located in the relatively nonbasic central region of histones the specific activities are about half those of the amino terminal area. No acetylation on chromatin of tyrosyl residues, located entirely in the nonbasic portions of the histones, has been observed. These data are consistent with models for nucleosome structure which envisage a protein core of the hydrophobic carboxyl terminal and central regions of the histones with the basic amino termini on the outside of the histone multimer, binding the nucleic acid.

The 5' termini of the DNA in monomeric HeLa cell nucleosomes can be stoichiometrically labeled with ^{32}P by transfer from ATP catalyzed by polynucleotide kinase. When digested with micrococcal nuclease, the 5' ends of the DNA of such particles become essentially totally acid soluble. The time course of digestion of 5' ends is much more rapid than that of bulk nucleosomal DNA. It appears that micrococcal nuclease degrades DNA within the nucleosome through an exonucleolytic mechanism. Digestion of 5' termini and whole nucleosomal DNA by DNAse I is, in contrast, identical in time course and extent. The DNA within nucleosomes has a potential, susceptible site for single-strand nicking by DNAse I every ten base pairs, shown by Noll and confirmed by others. Using nucleosomes labeled at the 5' end of the DNA we can now map the relative availabilities of these 13 potential attack sites along the DNA. Sites 20, 40 and 50 bases from the 5' terminus are preferentially cleaved. The central region of the DNA is almost totally resistant to nuclease scission, hardly any pieces 70 and 80 bases in length contain the 5' terminus of the original DNA.

Significance to Bio-medical Research and the Program of the Institute

Description of the structure of the eukaryotic nucleoprotein complex, chromatin,

is necessary for understanding the mechanism of gene regulation in higher organisms. Our current studies aim at complete understanding of the structural relationships between histones and DNA to create the nucleosome, the basic structure determining element of chromatin.

Proposed Course of Project: Investigations of histone-histone interactions and the mechanism of stabilization of DNA by its interaction with nucleosome cores will continue using techniques outlined above.

Publications:

Simpson, R. T. and Whitlock, J. P. Jr.: Chemical Evidence that Chromatin DNA Exists as 160 Base Pair Beads Interspersed with 40 Base Pair Bridges. Nuc. Acids Res. 3: 117-127, 1976.

Whitlock, J. P.Jr., and Simpson, R. T.: Removal of Histone H1 Exposes a Fifty Base Pair DNA Segment Between Nucleosomes. Biochemistry. (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15101-05 LNE

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

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

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

PI:	Robert T. Simpson	Surgeon, (R) (P)	LNE NIAMDD
OTHER:	Michael Bustin,	Visiting Scientist	LNE NIAMDD
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COOPERATING UNITS (If any)

Carnegie Institution of Washington, Department of Embryology

LAB/BRANCH
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SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

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

Hybridization of rRNA to chromatin fractions obtained by ECTHAM-cellulose chromatography failed to reveal large differences in gene dosage among the chromatin fractions which differ markedly in composition, structure and in vitro function Methodology for isolation of nuclei in non-aqueous media have been developed in order to allow determination of the ionic composition of the nucleus. This knowledge will allow further efforts at chromatin fractionation using the milieu existing in vivo, and hopefully reducing the chances of protein redistribution during prepartation of chromatin and its fractionation. A reproducible assay method for measurement of the binding of triiodothyronine to chromatin has been developed and is being employed to study transcriptional regulation by this hormone.

PHS-6040

Project Description

Objectives: To investigate the physical and chemical differences between portions of the eukaryotic genome which are transcribed and those which are repressed.

Methods Employed: Chromatin is isolated by conventional methods, sonicated to a size of about 1000 DNA base pairs and fractionated by ion-exchange chromatography on the weak anion exhcanger, ECTHAM-cellulose. Ribosomal DNA is isolated from *Xenopus laevis* by isopycnic centrifugation and a highly radioactive RNA copy (cRNA) made using *E. coli* DNA-dependent RNA polymerase. DNA isolated from column fractions is hybridized to this cRNA and the formation of stable hybrids assessed by filtration.

HeLa cells are grown in suspension culture in Eagle's spinner medium supplemented with horse serum. Cells are harvested, washed quickly with Hanks balanced salt solution and lyophilized exhaustively. The dry cells are disrupted by homogenization in cold anhydrous glycerol and nuclei prepared by sedimentation through a density gradient of 0-35% 1,3-chloropropanediol in glycerol. Various chemical constituents in these non-aqueous nuclei are measured: DNA, RNA and protein by standard chemical methods, Na and K by flame emission spectroscopy, Mg and Ca by atomic absorption spectroscopy and other trace elements by plasma emission spectroscopy using an instrument generously offered by the National Bureau of Standards. Contamination of the isolated nuclei by ionic constituents present in the washing medium is monitored by radioisotopic methods using $^{24}NaCl$.

Nucleosomes are prepared by isokinetic sucrose gradient centrifugation of micrococcal nuclease digested rat liver nuclei. Monomer nucleosomes, having a DNA chain length of about 180 base pairs, and a population of heavy nucleosomes, with average chain length of 1000 base pairs, are isolated. The transcription of these particles by *E. coli* DNA-dependent RNA polymerase is measured and compared with that of protein-free DNA. Initiation and propagation phases of the reaction are assessed and the size of the product RNA measured by gel electrophoresis and autoradiography using α-^{32}P-ATP.

Isolated chromatin, chromatin fractions, or nucleosomes are incubated at various concentrations and ionic conditions with ^{125}I-triiodothyronine, T3. Temperature and time of incubation are varied. Replicate tubes contain the same constituents plus a 200-fold excess of unlabeled T3 as a measure of non-specific binding of the hormone. At the conclusion of each incubation, a buffer containing 0.5% Tritium X-100 is added along with a measured quantity of hydroxyapatite. The tubes are centrifuged and washed several times with buffer. The final hydroxyapatite pellet is counted in a gamma spectrometer.

Major Findings: Hybridization of ribosomal cRNA to DNA fractions isolated from chromatin fractionated on ECTHAM-cellulose was attempted to localize "turned-on" genes in the elution profile. In contrast to previous results, where hybridization of globin cDNA to fractions from rabbit liver chromatin suggested that "turned-off" genes were concentrated in the mid-region of the elution

profile and thereby suggested that active chromatin would elute late, little difference in hybridization was seen. The amount of hybridizable RNA increased approximately two-fold from beginning to end of the elution profile. In part this might arise from the probing cRNA containing spacer region sequences, not transcribed *in vivo*, a conjecture currently under study by competing the non-spacer sequences with *in vivo* rRNA. Alternatively, the possibility exists that the globin results were spurious and these data, as well as those of others on integrated viral sequences, in fact demonstrate that chromatin fractionation by this and other techniques, while resolving physically and functionally different species, does not resolve DNA sequences which differ in their *in vivo* function.

Recently, evidence has been presented by Noll and coworkers that shearing methods in chromatin preparation lead to some damage to the structure of chromatin, possibly redistribution of DNA on histone nucleosome cores. Noll has proposed a chromatin preparation method based on micrococcal nuclease digestion of nuclei. While classical methods may damage structure, the method proposed by Noll is certain to destroy a portion of the chromatin - neither now appears to be satisfactory. Since many of the problems with proteins altering their binding to DNA may be a function of the ionic milieu and since difficulties in demonstrating fidelity of *in vitro* transcription to *in vivo* transcription might arise from altered binding of proteins in non-physiological salt conditions, we have begun efforts to define the ionic composition of eukaryotic nuclei. Nuclei isolated by non-aqueous methods should have no redistribution of small ionic components from the native *in vivo* condition. The ratios of protein, DNA and RNA in nonaqueous nuclei have been determined and agree well with literature values. Initially very high sodium concentrations were measured while potassium, calcium and magnesium contents were in a reasonable range. This was traced to contamination of the nuclear pellet by sodium from the washing medium. While less than 1.5% of the medium Na^+ was adsorbed onto nuclei and isolated with the nuclear fraction, this was sufficient to double the sodium content of the nuclear fraction. A simple technical change in the procedure apparently has removed this problem, allowing the further definition of the ionic composition of nuclei to proceed.

It now seems likely that the bulk of the DNA of a eukaryotic cell is complexed with histones in nucleosomes. It becomes of interest to see whether this histone bound DNA can be transcribed by RNA polymerase or whether histone-DNA dissociation is required for RNA formation. Transcription of the heavy nucleosome fraction was compared to that of monomer nucleosomes to assess the possible role of bridging DNA between nucleosomes in binding polymerase. In low salt both monomer and heavy nucleosome fractions are transcribed at low rates. Using the assay method of Cedar and Felsenfeld which permits separation of the initiation and propagation phases of RNA polymerase action, it can be shown that the block in transcription of nucleosome-bound DNA apparently exists at the level of propagation, although further studies using protein-free DNA of an equivalent size to that contained in the nucleosomes are required to make this conclusion firm.

One aspect of control of genetic activity in eukaryotes of particular interest is that mediated by hormones. Systems which respond to sex steroids

have been well studied by others with definition of the path of transport of the steroid to the nucleus, its binding as a complex with a cytoplasmic receptor protein to a chromatin component, likely an acidic protein, and synthesis of new mRNA under the aegis of the hormone. Of particular interest to us is the reported binding of triiodothyronine to chromatin, since no cytoplasmic receptor appears to be involved, enabling several forms of affinity chromatography to be employed to isolate potential regulatory regions of DNA or regulatory proteins. In beginning studies on T3 interactions with chromatin, we were frustrated by inability to repeat published results on binding using the assays employed by others. We have recently developed a binding assay which behaves properly and have begun to vary conditions to determine optimal conditions for the interaction of hormone and chromatin. Hydroxyapatite adsorbs chromatin and, in the presence of low concentrations of the nonionic detergent, Trixton X-100, does not bind T3 appreciably. Using the developed binding assay it has been possible to show 1) little temperature dependence of the binding, 2) saturation of competable T3 at a level of about 1000 sites per haploid equivalent for liver chromatin, 3) nearly theoretical competition of the competable binding of labeled T3 by cold T3, 4) ratios between *in vitro* binding of T3 to isolated chromatin and *in vivo* content of nuclear T3 for liver, kidney and testis which are equivalent (1:0.75:0.0). Further studies of T3-chromatin interactions are proceeding using chromatin prepared by different methods and fractionated by either sucrose gradient sedimentation or ECTHAM-cellulose chromatography.

Significance to Bio-Medical Research and the Program of the Institute. One of the most direct approaches to understanding the gross basis for transcriptional regulation in eukarytoic cells is study of the composition and structure of portions of the genome which are active as transcriptional templates and comparison with properties of repressed segments. In the past we have detailed the properties of segments of chromatin which are active as templates for RNA polymerase *in vitro* and compared them with inactive chromatin. We now seek to determine the proper ionic environment for further studies of chromatin purification, fractionation and *in vitro* functional assays in order to maintain native protein-nucleic acid interactions. While doing these studies, we continue to study transcription of chromatin and binding of regulatory molecules to chromatin, comparing several types of chromatin preparation to cover all bases. Our eventual goal is description of the mechanism of transcriptional regulation in animal cells - the features responsible for the stable differentiated state of a normal mature cell. Understanding of these features will facilitate understanding of neoplastic transformation and normal development and differentiation.

Proposed Course of Project Two major avenues for further investigation of structure-function relationships in chromatin will be pursued. First characterization of the normal ionic environment of the nucleoprotein will be made and methods developed for chromatin preparation and fractionation under physiologic conditions. Chromatin structure and *in vitro* function will be reassessed in the ionic conditions existing within the cell nucleus. Secondly, we will continue to study the triiodothyronine interactions with chromatin, measuring numbers and classes of sites, localizing binding to proteins or DNA, and attempting to isolate putative regulatory regions by affinity chromatographic methods.

Publications:

Simpson, R. T.: Chromatin Fractionation by Chromatography on ECTHAM-cellulose. In Stein, G., Stein, J. and Kleinsmith, L. (Eds.): Methods in Chromosomal Protein Research. Academic Press, New York, (in press).

Reeck, G. R.: Histone-DNA Interactions in Erythrocyte Chromatin. Arch. Biochem. Biophys. 172, 117-122, 1976.

Seale, R. L. and Simpson, R. T.: Effects of Cycloheximide on Chromatin Biosynthesis. J. Mol. Biol. 94, 479-501, 1975.

Simpson, R. T.: Distribution of Satellite DNA in Mouse Liver Chromatin Fractionated by ECTHAM-Cellulose Chromatography. Biochem. Biophys. Res. Commun. 65, 552-558, 1975.

Seale, R. L.: Assembly of DNA and Protein During Replication in HeLa Cells. Nature 255, 247-249, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15102-16 LNE

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Study of a Ribonuclease (barnase) and its Inhibitor (barstar) from Bacillus amyloliquefaciens.

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

PI:	Robert W. Hartley	Research Chemist	LNE NIAMDD
OTHER:	David M. Blow	Research Chemist	LMB, MRC
	Robert C. Sheppard	Research Chemist	LMB, MRC

COOPERATING UNITS (If any)

Large-scale Laboratory, Office of Chief, LNE, NIAMDD
Laboratory of Molecular Biology, Medical Research Council, Cambridge, ENGLAND

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland, 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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

Two proteins, barnase, the extracellular ribonuclease of Bacillus amyloliquefaciens, and barstar, its intracellular inhibitor, are being developed as a model system for the study of protein folding and protein-protein interactions. Amino acid sequence determination and X-ray structure characterization are being carried out along with thermodynamic studies of folding reactions. A peptide complementation system for barnase has been developed which will be used to investigate the roles of specific residues in both folding and enzymatic activity.

Project Description

Objectives: To further 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 structure, function and synthesis.

Specific objectives are: (1 the determination of the amino acid sequence and three-dimensional structure (by X-ray diffraction) of both proteins and the structure of their complex; 2) thermodynamic and kinetic analysis of structural transitions produced in both proteins by heat or denaturing reagents; 3) analysis of the folding and activity of both by means of peptide complementation systems analogous to those being used for the study of pancreatic ribonuclease and micrococcal nuclease; 4) all of the above with chemically and/or genetically modified sequences.

Methods Employed: B. amyloliquefaciens (strain H and derivatives and several others) are grown in synthetic media. Barnase is adsorbed from the culture medium onto phosphocellulose, Barstar is extracted from the cells. Their purifications make use of ion exchange, gel-permeation and affinity chromatography.

Work on the determination of amino-acid sequences involves amino acid analysis, enzymatic digestions, peptide separations and peptide sequencing by dansyl-Edman and difference-Edman techniques. Peptide separation and amino acid analysis are by automated ion-exchange chromatography using a modified JEOL analyzer equipped for detection with Roth's orthophthaldehyde reagent. Dansyl amino acids are identified by thin-layer chromatography on polyamide layers.

The reversible transitions undergone by barnase and barstar and their derivatives under the influence of temperature changes or denaturing agents are studied primarily by ultraviolet and fluorescence spectroscopy, but also by optical rotatory dispersion, circular dichroism, exclusion chromatography with the use of proteolytic enzymes and by equilibrium binding studies. These techniques are also used to study complementation reactions between natural and synthetic peptide sequences of the two proteins as well as conformational properties of each peptide alone. Studies of the peptide derivatives also include use of antibodies, chromatography, crystal growth, etc.

Crystals are grown in hanging drops (10 µl) suspended over suitable solvents. Isomorphous crystals containing heavy atoms are prepared by soaking crystals in solutions of appropriate heavy atom compounds. The three dimensional structure of barnase is being determined by methods of X-ray crystallography.

The Merrifield procedure has been used to synthesize portions of the barnase sequence.

Major Findings: Conditions have been found under which the barnase-barstar complex precipitates as crystals. The crystals are nicely shaped octahedrons, unrelated in shape to any barnase crystals. They are, as yet, too small for X-ray diffraction work.

It has been shown that the barnase peptide bar (88-110) complements bar (1-102) by binding, at 25°, with a dissociation coefficient of 6×10^{-12} M, yielding a complex with 60% of the ribonuclease activity of native barnase. Bar (88-110) includes the C-terminus. Bar (99-108) also complements bar (1-102) but with a dissociation coefficient several orders of magnitude greater. Bar (103-110) does not complement.

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 make it an excellent choice for a long-range study of the relation between the sequence of an enzyme and its structure and function. 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.

It has been suggested that protein-detergent interactions may serve as models for interactions between proteins and phospholipids in biological membranes.

Proposed Course of Project: Collaboration with the workers in Cambridge on the structure of barnase and on peptide complementation will continue. Our current small stock of barstar will be dedicated to barnase-barstar crystal growing for the present time. Barstar sequencing will resume when a sufficient backlog is available.

Publications:

Hartley, R. W.: A Two-State Conformational Transition of the Extracellular Ribonuclease of Bacillus amyloliquefaciens (Barnase) Induced by Sodium Dodecyl Sulfate. Biochemistry 14, 2367-2370, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15103-04 LNE

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biochemistry of the Pituitary Glycoprotein Hormones.

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

PI:	Peter G. Condliffe	Research Chemist	LNE	NIAMDD
OTHER:	Kenji Sorimachi	International Fellow	LNE	NIAMDD

COOPERATING UNITS (if any)

Department of Physical Biochemistry, Institute of Endocrinology, Gunma University, JAPAN

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

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

The subunits of b-TSH were separated and purified by an improved two stage gel electrophoresis system. Conditions for reduction of the disulfide structure of b-TSH were reinvestigated to find the optimal conditions for preparation of the subunits. During these studies certain prepartions which had lost most of their biopotency was found to contain nicked peptide chains yielded low molecular weight fragments that could be demonstrated by SDS gel electrophoresis.

PHS-6040

Project Description

Objectives: During the past decade, the subunits of the pituitary hormones, TSH, LH and FSH have been isolated from human glands and from several species of animal glands. As a result, the peptide sequences are known for the subunits of b-TSH, b-LH and b-FSH. Partial information from peptide mapping of the human hormones, together with recombination experiments involving their various subunits strongly suggest that the structures of h-TSH and h-LH are very close to those of the bovine.

The demonstration in several laboratories that the glycoprotein hormones from the pituitary and also from the placenta, possess 2 subunits, not linked by disulfide or other covalent bonds, raises several questions about their synthesis and the regulation of their secretion.

1. Are the subunits, which each have between 100 and 120 amino acid residues, synthesized separately and then combined to yield the active hormone or are they derived from a single pro-hormone as is the case with insulin.

2. If the subunits are synthesized separately, are there, in fact, two pro-hormones each of which is activated and which then are combined to yield the active hormone?

3. Do the pituitary proteolytic enzymes play a role in the secretion and activation of these hormones?

Methods Employed: Against this background, a detailed investigation of the pituitary proteolytic enzymes has been started. Their role in the conversion of putative pro-hormonal forms of the glycoprotein hormone to the active forms found in the pituitary and the circulation is being studied using bovine, salmon and human glands.

Proteolytic activity is assayed by the method of Anson using denatured human hemoglobin prepared from out-dated blood from the NIH blood bank.

The glycoprotein hormones and their derivatives are identified by radioimmunoassay, gel electrophoresis, and their behavior in gel filtration and other techniques.

Glycoprotein hormone subunits are prepared by gel filtration or by a new gel electrophoresis technique developed in this laboratory.

Results: Purified preparations of bovine TSH were prepared using affinity chromatography in combination with the classical techniques of gel filtration and ion exchange chromatography. These preparations were compared with older preparations prepared during the last ten years which had retained their biological activity.

SDS gel electrophoresis was used to reinvestigate the conditions under which b-TSH dissociates into its constituent subunits. The effects of SDS

itself, urea, pH and temperature were assessed by the decrease in the amount of undissociated TSH and the increase in the dissociated components as revealed by SDS gel electrophoresis. In SDS alone 25%-30% dissociation occurred. Pretreatment with increasing concentrations of urea up to 7.1 M increased the degree of dissociation to 70% after 4 hours at room temperature. Maximum dissociation was achieved by treatment at pH 2.5 and 37° C for one hour. In 1 M propionic acid approximately 10% of the TSH was still undissociated after 18 hours at 25° C.

SDS gel electrophoresis was then used to investigate the relationship between the reduction of the disulfide bonds and the dissocation into its subunits.

SDS inhibits the effect of urea on the dissociation of b-TSH. The inhibition by SDS is gradually overcome in the presence of increasing concentrations of the reducing agent. When the reduction of b-TSH was carried out in 6 M urea without SDS, aggregated material appeared whose molecular weight was higher than that of the undissociated b-TSH. However, dissociation of b-TSH into subunits occured at low concentrations of DTT alone.

DOC also inhibits both the dissociation of b-TSH in urea and the reduction of the disulfide bonds in b-TSH. Disruption of the disulfide structure of one preparation by reduction not only caused disaggregation but also gave rise to low molecular weight components. This result suggested that there were breaks or "nicks" in the subunits. Subsequent bioassay showed that this particular preparation had lost most of its biological activity compared with other preparations which displayed the usual TSH_{α} and TSH_{β} bands in SDS gel electrophoresis after reduction.

A two stage electrophoretic method was worked out for the separation of glycoprotein hormones and for histones. SDS gel electrophoresis was carried out as the first step. The buffer containing SDS was replaced after 1 hour by a buffer with no SDS and electrophoresis was continued. As SDS was eluted from the gel into the lower buffer vessel, the mobilities of the various protein components distributed in the gel by the first stage, were altered. The subunits of b-TSH were significantly separated by this two stage procedure which could be used for their purification. The components of calf thymus histone could also be separated by this technique.

Proposed Course of Project: The principal investigator on this project has recently resigned from NIAMDD to take a science-administrator position in FIC. The project will therefore be discontinued. Certain aspects of the work will be continued in other laboratories including the collaborating Japanese laboratory at Gunma University.

Publications:

NONE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15104-04 LNE

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Preparation of Large Oligonucleotides from PUrified Nucleic Acids.

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

PI:	George Rushizky	Research Chemist	LNE NIAMDD
OTHER:	David L. Rogerson, Jr.	Research Chemist	LNE NIAMDD

COOPERATING UNITS (If any)

Large-Scale Laboratory, Pilot Plant, LNE,NIAMDD

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	1.1	1.0

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

Hydrolysis of nucleic acids with restriction endonucleases will be used for the preparation of mg amounts of large oligonucleotides suitable for studies of their chemical, enzymological, and biological properties.

Project Description

Objectives: To isolate and/or develop procedures for the preparation and isolation of large oligonucleotides (fragments of nucleic acids of chain lengths larger than 10) in preparative (mg) amounts, using in part the facilities of the Pilot Plant, LNE. The oligomers will be used for chemical, enzymological and biological studies.

Methods Employed: Phages T7 ϕ 80 and PM2 were grown and their DNAs isolated by standard procedures. The DNAs were hydrolyzed by restriction endonucleases isolated in the lab or available commercially. For quantatition of the yields of oligomers, ^{32}P-labeled DNAs were employed. Methods for the analytical and/or large-scale isolation of oligonucleotides, such as column chromatography on DEAE-cellulose, gel filtration on agarose beads and polyacrylamide gel electrophoresis (PAGE) were investigated.

Major Findings: First attempts to purify Bam I nuclease from Bacillus amyloliquefaciens using T7 DNA as substrate failed. The enzyme was then found not to cleave T7 DNA. This result was unexpected, but Eco RI, another 6-base specific restriction nuclease, also does not hydrolyze T7 DNA (results of P. Leder). Bam I was purified using PM2 DNA which is cleaved twice, since this DNA was more readily prepared than ϕ 80 DNA. Problems encountered with the growth of PM2 were eventually solved by avoidance of glassware washed with detergents, which is in line with the presence of lipids in PM2. The purified Bam I was free of phosphatase, exonuclease and other DNAse activities. Its activity (or lack of it) toward DNA from PM2, T7 and ϕ 80 agreed with that observed with commercially available Bam I. Because of high cost, the latter enzyme is not suitable for the preparation of mg amounts of oligomers: 100 units (to hydrolyze 20 mg DNA) cost $99, while 10 liters of B. amyloliquefaciens (40 g of cells) yield about 2×10^6 units of purified enzyme. Similar considerations apply to other restriction enzymes, ligases, and nucleic acids.

To obtain oligomers in the range of 150-200 base pairs, T7 DNA was used because it is readily prepared in the pilot plant (5-10 g of phage per 200 liter of lysates). Of 8 commercially available enzymes tried, the best results (single rather than clustered bands of oligomers of desired chain lengths) were obtained with Hha I of GCG/C specificity, followed by Hae III (GG/CC), Hpa II (C/CGG) and Alu I (AG/CT).

Work is in progress on the purification of these and related enzymes. Fractionation of oligomers without ^{32}P label by PAGE is difficult to scale up. Nevertheless, separation of such cleavage products of nucleic acids is at present almost exclusively done by PAGE, because minor differences between DNA segments can be detected even such as small deletions, substitutions, additions or rearrangements not detectable by methods such as E-M mapping or hybridization.

To use column methods for the preparative isolation of large oligonucleotides, a small number of single oligomers should differ from each other maximally in chainlength, which leads back to a suitable choice of enzyme and DNA substrates. Studies carried out so far with column methods await a larger catalog of oligomers than available now.

Work with S_1 nuclease on the hydrolysis of viral and transfer nucleic acids has been concluded (See last report).

Significance to Bio-Medical Research and the Program of the Institute. Availability of large oligomers in purified form will facilitate projects, also in collaboration with others, that require these compounds in mg amounts and in unlabeled form. For example, isolation of 140-200 base pair DNA fragments may be useful for studies on the assembly of nucleosomes. Other projects involve speculation, such as the possible formation of antibodies to large oligomers after their denaturation to single-stranded compounds. Other areas include studies on the template properties of DNA fragments for synthesis of RNA or DNA by such enzymes as E. coli polymerase or DNA polymerase I, for hybridization to transcripts and binding to specific proteins or stable RNA genes. In higher organisms, question regarding the size and frequency of repetitive DNA may be aided by the availability of large oligomers, and determination of the site of origin of DNA fragments in the genome by hybridization to chromosome preparations may be facilitated.

The PM2 - Bam I system may offer advantages over the E. coli systems proposed at present for cloning of recombinant DNA molecules. Being a marine virus, PM2 and its host, the cryophilic Pseudomonas BAL 31 do not grow at temperatures above 29°. While little is known about PM2 genetics, it is less likely to mutate back to higher temperatures than low temperature E. coli mutant vectors.

Proposed Course of Project: The long range purpose involves the understanding of the genome arrangement of higher organism and the isolation of specific fragments of chromosomes. To begin with, 140-200 base pair fragments of T7 DNA and PM2 halves will be prepared.

Publications:

Rushizky, G. W. and Mozejko, J. H: Strip counting of ^{32}P in gel electrophoresis strips. Anal. Biochem. 65, 540-542, 1975.

Rushizky, G. W., Shaternikov, V. A., Mozejko, J. H. and Sober, H. A.: S_1 nuclease hydrolysis of single-stranded nucleic acids with partial double-stranded configuration. Biochemstry 14, 4221-4226, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 15200-16 LNE

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies on Folic Acid

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

PI:	Bernard T. Kaufman	Research Chemist	LNE NIAMDD
OTHER:	Verne F. Kemerer	Staff Fellow	LNE NIAMDD

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Vitamin Metabolism Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

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

Electrofocusing of chicken liver dihydrofolic reductase purified by affinity chromatography on a Methotrexate-Sepharose column reveals multiple peaks of enzyme activity. The major peak is the "pure" enzyme devoid of bound substrate. At least two minor peaks may contain bound dihydrofolate and TPNH. The properties of the homogeneous-substrate free enzyme have been examined by amino acid analysis, sulfhydryl group titration, methotrexate titration, ultracentrifugation, circular dichroism and fluorescence spectroscopy.

PHS-6040

Project Description

Objectives: Dihydrofolic reductase is an enzyme concerned with the conversion of the essential viatamin folic acid to the coenzymatically active tetrahydrofolic acid. In addition, dihydrofolic reductase is believed to be the site of action of one of the most effective cancer chemotherapeutic drugs, methotrexate. Thus it is the purpose of this project to isolate and determine the biochemical and molecular properties of dihydrofolic reductase from liver in order to gain insight at the molecular level of the interaction of this enzyme with both its normal substrates and drugs.

Methods Employed: Affinity chromatography using a chromatographic system based on the drug methotrexate coupled to sepharose has been used to obtain dihydrofolic reductase from liver extracts. Further purification and characterization involved the use of isoelectric focusing. Additional methods of chemical, enzymatic, and physical analysis involved kinetic measurements by spectrophotometry, ligand binding by spectrofluorometry, molecular weights and hydrodynamics by ultracentrifugation, and molecular conformation by circular dichroism.

Major Findings: Chicken liver dihydrofolic reductase apparently purified to homogeneity by affinity chromatography on a Methotrexate-Sepharose column contains variable amounts of tightly bound dihydrofolic acid. The most effective method for removal of the bound substrate has been found to be isoelectric focusing. Furthermore this procedure also removes unknown contaminants as evidenced by the appearance of bands of precipitate not associated with the enzyme. In addition, the isoelectric profile revealed as many as three distinct peaks of enzyme activity. The major peak which represents 65 to 80% of the total activiy exhibits an isoelectric point of 8.4. A second peak of activity is observed at an isoelectric point of 7.4 and finally a small peak representing less than 5% f the total activity is observed at 6.8.

The enzyme obtained from the major isoelectric band is devoid of bound substrate, exhibits a 280/260 ratio approaching 2, and a specific activity of approximately 14 μmoles substrate reduced per min per mg protein which is about 20% higher than previous samples.

In view of the very tight binding of the cosubstrates, TPNH and dihydrofolate reductase, the effect of the addition of the ligands on the Isoelectric point was examined in order to ascertain if the two additional peaks of the enzyme may be due to the effects of bound substrate. Readdition of excess TPNH to the apoenzyme did shift the position of the enzyme from an isoelectric point of 8.4 to 5.8. Thus, neither of the two minor peaks is due to the binding of TPNH. On the other hand, the readdition of excess dihydrofolate not only did not affect the isoelectric point, but the added ligand was completely removed from the enzyme during isoelectric focusing. In fact, added methotrexate was also removed from the enzyme during isoelectric focusing.

However, spectral examination of the enzyme obtained from the 7.4 peak indicates that it does contain bound dihydrofolate. Furthermore, reisoelectric focusing the 7.4 peak results in its shift to the position characteristic of

the apoenzyme, 8.4. Thus it is concluded that the pH 7.4 peak does indeed contain bound dihydrofolate and this is only observed when the protein content is approaching the capacity of the isoelectric band on the column. Traces of activity remaining in the 7.4 band as well as the 6.8 band may be due to deamidated enzyme.

Additional molecular parameters of chicken liver dihydrofolic reductase are being examined. Thus far it has been determined that the mol. wt. is 22,500 ± 480 confirming the unusually low molecular weight for this pyridine nucleotide enzyme. The molar extinction coefficient is 26,690 and the N-terminal amino acid is valine.

The interaction of substrates with the enzyme was measured by determination of the dissociation constants by fluorescence quenching: TPNH, 3.1×10^{-7}; TPN, 3.8×10^{-6} dihydrofolate, 1.4×10^{-8}; folate, 6.7×10^{-8}; and the inhibitor methotrexate, 2.8×10^{-9}. These values are approximately 10-15x smaller than the corresponding K_m's and confirm the tightness of the binding of substrates to chicken liver dihydrofolic reductase. The enzyme reacts rapidly with p-hydroxymercuribenzoate. Direct titration with this reagent reveals the presence of one sulfhydryl group. Although the reaction with dithionitrobenzoate is quite slow, again only one sulfhydryl group is evident. The presence of one cysteine is revealed by amino acid analysis confirming the observation that only one SH is present.

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 nucleic acid and the chief site of action of certain cancer chemotherapeutic agents. Thus the understanding of the structure and function of this enzyme would be of basic importance in both normal metabolism and in cancer treatment.

Proposed Course of Project: Now that the basic properties of the homogeneous protein are known for both the beef and chicken liver dihydrofolic reductase, studies will be directed toward an investigation of the binding of substrates and drugs to these enzymes. In addition, the role of the various functional groups, i.e. sulfhydryl, of the enzyme in both binding and catalytic activity will be studied.

Publications:

Kaufman, B. T. and Kemerer, V. F.: Purification and Characteristics of Beef Liver Dihydrofolic Reductase. Arch. Biochem. Biophys. 172, 289, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 15300-11 LNE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biochemical Studies Related to Nutrition

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

PI: J. G. Bieri, Chief, Nutritional Biochemistry Sec., LNE, NIAMDD

Other: G. L. Catignani, Staff Fellow, Nutritional Biochemistry Sec., LNE NIAMDD

S. L. Thorp, Chemist, Nutritional Biochemistry Sec., LNE, NIAMDD

COOPERATING UNITS (if any)

Dr. J. J. Gart, NCI
Dr. K. Ahmad, University of Dacca, Bangladesh

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Nutritional Biochemistry Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	2	3

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

Studies of vitamin E nutrition and biological function have led to the discovery of a binding protein for α-tocopherol in rat liver supernatant. In other studies, α-tocopherol and γ-tocopherol were compared for their ability to prevent lipid peroxidation in the red cell membrane. α-Tocopherol was 38% as active as γ-tocopherol.

PHS-6040
(12-75)

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.

Major Findings - Studies from this laboratory have demonstrated that rat liver cytoplasm contains a protein which binds [^{3}H] α-tocopherol with a high affinity and specificity. Incubations of high speed supernatant with [^{3}H] α-tocopherol *in vitro* followed by centrifugation on 5-20% sucrose gradients resulted in a peak of bound radioactivity which sedimented in the 3S region of the gradient. In close agreement with the S value, gel filtration yielded an estimated molecular weight of 31,000. Binding was completely abolished by incubation of labeled supernatant with Pronase indicating a protein character of the binding component.

The presence of a 400 fold excess of unlabeled tocopherol reduced binding of [^{3}H] α-tocopherol by 95% or greater while a 400 fold excess of α-tocopheryl acetate or α-tocopheryl quinone had no effect on binding. Specificity was further demonstrated by the fact that a wide variety of other lipid soluble compounds including 6-hydroxy, 2, 5, 7, 8-tetramethyl chroman-2 carboxylic acid (α-tocopherol minus the side chain), retinol, retinoic acid, 1, 25 dihydroxyvitamin D_3, ubiquinone 9, vitamin K, oleic acid and cholesterol did not compete for binding sites. A high affinity can be inferred from the persistence of bound counts following both sucrose gradient centrifugation and gel filtration.

The binding protein exhibited a finite number of binding sites as judged by the ability of excess tocopherol to reduce binding of labeled tocopherol and rough estimates indicated a K_d of $2\text{-}4 \times 10^{-8}$M. Neither salt (.2M KCl) nor temperature affect maximal binding or sedimentation behavior. Optimal binding conditions were achieved by incubation at 25°C for 4 hrs at pH 7.5. The binding protein is relatively heat stable, losing only 8% binding activity at 46°C for 2 hr. Examination of rat tissues including kidney, heart, lung, testis, intestinal mucosa, muscle and liver demonstrated that the binding protein was detectable by present assay methods only in the liver.

In other studies, the antioxidant activities of α-tocopherol and γ-tocopherol in protecting the red cell membrane were determined. The tocopherols, labeled with ^{14}C, were incorporated into the red cell membrane by incubating cells with solutions of the tocopherols in bovine albumin.

The cells were washed and subjected to peroxidative hemolysis by dialuric acid. Analyses of variance of the response curves (percent hemolysis vs. red cell tocopherol content) revealed that γ-tocopherol had 38% of the activity of α-tocopherol. This contrasts with 10% biological activity in preventing vitamin E deficiency symptoms when γ-tocopherol is fed in the diet. No evidence was found for an interaction between the two tocopherols when present in the red cell membrane simultaneously.

As part of a PL-480 project with the University of Dacca, samples of rice from three provinces of Bangladesh were analyzed for their selenium content by both animal bioassay and chemical analysis. Bangladeshi rice by both assays had only one-sixth to one-third the selenium content of U.S. rice.

Publications:

Bieri, J. G. & Evarts, R. P.: Effect of Plasma Lipid Levels and Obesity on Tissue Stores of α-Tocopherol. Proc. Soc. Exp. Biol. & Med. 149: 500-502, 1975.

Bieri, J. G.: Vitamin E. Nutr. Rev. 33: 161-167, 1975.

Bieri, J. G. & Evarts, R. P.: Tocopherols and Polyunsaturated Fatty Acids in Human Tissues. Am. J. Clin. Nutr. 28: 717-720, 1975.

Farrell, P. M. & Bieri, J. G.: Megavitamin E Supplementation in Man. Am. J. Clin. Nutr. 28: 1381-1386, 1975.

Bieri, J. G., Evarts, R. P. & Gart, J. J.: Relative Activity of α-Tocopherol and γ-Tocopherol in Preventing Oxidative Red Cell Hemolysis. J. Nutr. 106: 124-127, 1976.

Bieri, J. G. & Ahmad, J.: Selenium Content of Bangladeshi Rice by Chemical and Biological Assay. J. Agri. & Food Chem. (in press).

Catignani, G. L.: An α-Tocopherol Binding Protein in Rat Liver Cytoplasm. Biochem. & Biophys. Res. Comm. 67: 66-72, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z 01 AM 15302-06 LNE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biochemical Studies of Hepatic and Intestinal Function

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

PI;	Dr. H. G. Windmueller	Research Chemist	LNE	NIAMDD
OTHER;	Dr. L. M. Pinkus	Staff Fellow	LNE	NIAMDD
	Mr. A. E. Spaeth	Chemist	LNE	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Nutritional Biochemistry Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

SUMMARY OF WORK (200 words or less - underline keywords) The metabolic activity of the small intestine in vivo is being studied in an auto-perfused preparation of rat jejunum in situ recently developed in this laboratory. The rates of uptake and the metabolic fate of nutrients taken up by intestine from the blood or absorbed from the lumen are being quantitatively determined by enzymatic, chromatographic, and radiochemical analyses. Of recent interest are the following: (a) The remarkably high rate of plasma glutamine metabolism; (b) the low rate of plasma glucose metabolism in vivo as compared with rates in vitro; (c) the extensive metabolism by intestine of several dietary amino acids absorbed from the lumen, including glutamine, glutamate, aspartate, and arginine. In related studies, the enzymes of glutamine metabolism in intestine are under investigation. A highly-active phosphate-dependent glutaminase has been localized in mitochondria of both villous and crypt cells from all regions of the small intestine. The development of the enzyme is being investigated and its properties determined, including its response to inhibitors that may be useful in determining the role of glutamine in this tissue.

PHS-6040
(12-75)

Project Description:

Objectives: To increase our understanding of the biochemical transformations occurring in the liver and small intestine. To define the nutritional requirements of these organs, to study the effects of hormones and nutritional variables, and to study the physiological relationship of these organs to other tissues in the body.

Methods employed: Surgical and biochemical techniques are used. Biochemical determinations are made on rat livers and preparations of small intestine vascularly perfused with blood under physiological conditions. Also used are rats surgically prepared with indwelling lymph or bile cannulae as well as a supra-diaphragmetic rat preparation. The metabolic and transport activity of the organs is assessed by the use of radioactive isotopes and measurements of tissue and blood enzyme and metabolite concentrations. Tissue enzymes are localized and characterized.

Major Findings: Studies are continuing to elucidate the major metabolic pathways in the small intestine in vivo, by quantitating the uptake rate and metabolic fate of the major metabolic substrates utilized by intestine from both the blood and the gut lumen. These studies are made possible by use of an auto-perfused preparation of rat jejunum in situ developed recently in this laboratory. Previous studies with this preparation revealed that large quantities of plasma glutamine are metabolized by the small intestine in addition to dietary glutamine and glutamate absorbed from the lumen. The carbon from these amino acids is released into the blood as CO_2, organic acids, and other amino acids (citrulline, proline), and the nitrogen mostly as alanine, ammonia and citrulline.

These studies have now been extended to include other dietary amino acids. L-[U-^{14}C]aspartate, luminally administered alone (6 mM) or together with 18 other amino acids plus glucose, was absorbed much more rapidly than glutamate, but as with glutamate, less than 1% was recovered intact in intestinal venous blood. About 50% of the aspartate carbon was recovered in CO_2, 24% in organic acids, mostly lactate, 12% in other amino acids (alanine, glutamate, proline, ornithine and citrulline), and 10% in glucose, apparently the first demonstration of intestinal gluconeogenesis in vivo. A sensitive and specific enzymatic assay was developed to quantitate the ^{14}C-labeled glucose produced. In contrast to absorbed aspartate and glutamine, nearly all L-asparagine was absorbed intact, less than 1% being catabolized. About 4% of the absorbed dose was incorporated into the tissue protein, as was the case with all the amino acids studied. With L-arginine, only 60% of the luminally-administered dose was absorbed intact while 33% was hydrolyzed to ornithine and urea. The urea and 38% of the ornithine were released into the blood; the remaining ornithine was metabolized further by intestine to citrulline, proline, glutamate, organic acids and CO_2. Thus catabolism of several dietary amino acids absorbed from the gut lumen may provide an important energy source in small intestine of fed rats.

Oxygen consumption and CO_2 production by small intestine in vivo were determined with the same preparation, as well as the CO_2 contribution from

several plasma substrates. In the jejunum of fasted rats metabolism of plasma glutamine accounts for about 40% of the total CO_2 produced. Utilization of glucose, generally regarded as the major oxidative substrate for gut, was less than 50% that of glutamine on a molar basis and accounted for only 4% or less of the CO_2 produced.

We have also investigated the activity of several glutamine-degrading enzymes in intestine. Mucosal homogenates contain at least 10-fold more phosphate-dependent glutaminase than necessary to account for the observed rate of glutamine metabolism (3-8 μmoles glutamate formed/mg protein/h). Activity is virtually uniformly distributed along the duodenum, jejunum, and ileum. Almost no activity is found in the stomach and little in the cecum and colon. At least 90% of the activity in small intestine is confined to mucosal epithelial cells. Specific activity is similar in epithelial cells isolated sequentially from villous tips to crypts, i.e. at all stages of cell maturation. Subcellularly, glutaminase is associated with mitochondrial membranes. The glutamine analogs, 5-chloro-4-oxo-L-norvaline and 6-diazo-5-oxo-L-norleucine strongly inhibit glutaminase activity in isolated mitochondria or incubated intestinal segments. Glutamine protects against this inhibition. Other glutamine-utilizing enzymes, including glutamine transaminase, "phosphate-independent glutaminase" and several amidotransferases, exhibit extremely low activity when compared to glutaminase. Available data indicate that glutamine hydrolysis catalyzed by mitochondrial phosphate-dependent glutaminase in mucosal epithelial cells is quantitatively the major pathway of glutamine metabolism in intestine.

Significance to Biomedical Research and the Program of the Institute: These studies contribute basic information on the metabolism of the intestine and on the digestive process under physiological conditions in the intact organ. They have revealed the relative quantitative importance of glutamine as a respiratory substrate in this tissue, and have permitted the identification and quantification of products resulting from the intestinal metabolism of a variety of dietary amino acids. The localization and partial characterization of a quantitatively important enzyme in intestine was achieved and inhibitors of the reaction were found which may prove useful in determining its physiological significance.

Proposed Course: This unique intestinal preparation will be used to identify other nutrients, both dietary and from the circulation, that undergo metabolism in the intestine.

Publications:

Windmueller, H. G. and Spaeth, A. E.: Intestinal Metabolism of Glutamine and Glutamate from the Lumen as Compared to Glutamine from Blood. Arch. Biochem. Biophys. 171: 662-672, 1975.

Windmueller, H. G. and Spaeth, A. E.: Metabolism of Absorbed Aspartate, Asparagine and Arginine by Rat Small Intestine in vivo. Arch. Biochem. Biophys. (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM 15400-02-LNE

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Hormones, lipoprotein lipase and fat metabolism

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

PI: Robert O. Scow, Chief, Section on Endocrinology, LNE NIAMDD
Sidney S. Chernick, Scientist Director, LNE NIAMDD
OTHER: Peter M. Spooner, Staff Fellow LNE NIAMDD
Angelo A. Ucci, Jr., Research Associate LNE NIAMDD

COOPERATING UNITS (If any)
Z01-AM 15401-04

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Endocrinology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	2	4

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

The main objectives of the project are: (1) Determine the effect of hormones and other factors on the metabolism of lipid in adipose tissue, mammary gland, crop sac and other tissues. (2) Determine how prolactin and other hormones regulate lipoprotein lipase activity in tissues. (3) Determine the role of lipoprotein lipase in the uptake of triacylglycerol, cholesterol and other lipids from chylomicrons. (4) Determine the role of apolipoproteins in the transport and uptake of triacylglycerol in blood.

PHS-6040
(12-75)

Project Description:

Objectives: To determine the influence of hormones, lipoprotein lipase and other factors on the metabolism of fat in liver, adipose tissue, lactating mammary gland, crop sac and other tissues. To determine how hormones regulate lipoprotein lipase activity in tissues.

Methods Employed: Animals are deprived of one or several endocrine glands and treated with hormones or other substances. The effects of the above on lipid metabolism and lipoprotein lipase activity in different tissues are determined with in vivo and in vitro studies using conventional and radioisotopic techniques. Effect of hormones added in vitro are also studied.

Major Findings: Mechanism of hormonal regulation of lipoprotein lipase activity in mammary gland. Lipoprotein lipase activity is increased in mammary gland and decreased in adipose tissue during lactation. Earlier studies showed in lactating rats that these changes in enzyme activity are mediated through prolactin secretion by the anterior pituitary gland. The present study was initiated to determine the mechanism of hormonal regulation of lipoprotein lipase activity in mammary gland.

Preliminary findings reported last year suggested that factors in addition to prolactin may be needed for stimulating lipoprotein lipase activity in mammary gland of immature rats. Recent studies in pregnant rats showed that lipolytic activity in mammary tissue on the 16th and on the 20th days of gestation was unaffected by prolactin injected for 2-1/2 days. A three-fold increase in enzyme activity of mammary tissue was obtained on the 20th, but not on the 16th, day of gestation when prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was injected for 2-1/2 days. $PGF_{2\alpha}$ also reduced serum triacylglycerol concentration 50% on the 20th but not the 16th day. Analyses of serum showed that $PGF_{2\alpha}$ reduced progesterone concentrations >80% on both days and increased immunoreactive prolactin concentrations 5-fold on the 20th day, but had no effect on prolactin concentrations on the 16th day. These findings suggest that the surge in serum $PGF_{2\alpha}$ prior to parturition on the 21st-22nd day of gestation may be important in the regulation of progesterone and prolactin concentrations in serum, and thereby affect prolactin-dependent systems. The possibility that lipoprotein lipase activity in mammary tissue may be unresponsive to prolactin in the presence of high concentrations of progesterone in serum is being studied in vivo in 20-day pregnant rats and in vitro in cultured rat mammary epithelial cells.

Uptake of triacylglycerol and cholesterol from blood by perfused lactating rat mammary tissue. Earlier studies showed that perfused mammary tissue of lactating rats takes up and hydrolyzes triacylglycerol from chylomicrons in blood, and that this process is dependent on lipoprotein lipase activity in the capillaries. Lactating mammary tissue also removes triacylglycerol from artificial emulsions but only if they are first incubated with serum. Experiments are in progress to determine the component(s) of serum necessary for uptake of triacylglycerol by perfused mammary tissue. Studies

by others showed that hydrolysis of triacylglycerol by lipoprotein lipase in vitro requires apolipoprotein C-II, a normal constituent of chylomicrons, very low density lipoproteins and high density lipoproteins (HDL) of serum. Preliminary findings indicate that triacylglycerol in artificial emulsions is taken up and hydrolyzed by perfused mammary tissue if the emulsion is incubated priorly with human HDL. It is not known yet which component of HDL is involved.

Studies elsewhere have shown that 50-85% of the cholesterol secreted in milk is derived from blood and that cholesterol is readily taken up from chylomicrons by lactating mammary tissue. Recent studies in perfused mammary tissue showed that chylomicron cholesterol is taken up with triacylglycerol and that uptake of both lipids is markedly suppressed when lipoprotein lipase activity is low in the tissue. It is proposed that uptake of triacylglycerol by the tissue requires the action of lipoprotein lipase, whereas uptake of cholesterol is secondary to reduction of the triacylglycerol core, through the action of the enzyme on the core and transfer of the product to tissue and blood.

Effect of prolactin on lipoprotein lipase activity and uptake of blood triacylglycerol in crop sac of pigeons. Prolactin stimulates in pigeons growth of the crop sac and formation of crop "milk", which consists of desquamated epithelial cells containing 12% fat. Last year it was reported that prolactin injections increase markedly lipoprotein lipase activity in crop sac, suggesting that blood triacylglycerol might be a source of fatty acids for the formation of crop "milk". Recent studies showed that uptake of triacylglycerol from both injected chylomicrons and injected artificial emulsions increased with lipoprotein lipase activity in crop sac of pigeons treated with prolactin. Most of the fatty acids retained were present as triacylglycerol, 6% as diacylglycerol, and lesser amounts (<3% each) as monoacylglycerol, free fatty acids and phospholipids. Preliminary radioautographic findings indicate that some of the fatty acids taken up were present, within 10 min, in the cytoplasm and lipid droplets of basal epithelial cells of the crop sac. The results show that blood triacylglycerol is a source of fatty acids for crop sac epithelium, and that lipoprotein lipase is involved in the uptake of blood triacylglycerol by this tissue.

Significance to Biomedical Research and the Program of the Institute. Lipoprotein lipase is necessary for uptake of triacylglycerol from blood by extrahepatic tissues. Earlier studies in this laboratory showed that the enzyme acts in capillary endothelium to hydrolyze triacylglycerol to partial glycerides and fatty acids which cross the capillary to be utilized by tissue cells. More recent studies showed that uptake of chylomicron cholesterol by extrahepatic tissues is also dependent on lipoprotein lipase activity. We have proposed a model, based in part on the above findings, for transport of partial glycerides, fatty acids and cholesterol from chylomicrons to cells in tissue by lateral diffusion in a continuous water-lipid interfacial plane consisting of the chylomicron surface film and the external leaflets of plasma and intracellular membranes of endothelial, interstitial and parenchymal cells (see project Z01-AM 15401-04 for other details of model). It is

also proposed that lipids derived from chylomicrons leave the interfacial continuum in parenchymal cells where they are esterified, to either triacylglycerol or cholesterol ester, and separate from the lipid interface to accumulate between the external and cytoplasmic leaflets of endoplasmic reticulum. It is likely that this mechanism for transport and accumulation of lipid in extrahepatic tissues operates in the walls of large blood vessels.

Proposed Course. The mechanism of action of prolactin in the regulation of lipoprotein lipase activity will be studied in rodent mammary tissue and in pigeon crop sac. The role of various apolipoproteins in the transport and uptake of triacylglycerol from blood will also be studied.

Publications:

Garrison, M.M. and Scow, R.O.: Effect of prolactin on lipoprotein lipase in crop sac and adipose tissue of pigeons. Am. J. Physiol. 228: 1542-1544, 1975.

Scow, R.O., Blanchette-Mackie, E.J. and Smith, L.C.: Role of capillary endothelium in the clearance of chylomicrons: A model for lipid transport from blood by lateral diffusion in cell membranes. Circ. Res. In press.

Zinder, O., Mendelson, C.R., Blanchette-Mackie, E.J. and Scow, R.O.: Lipoprotein lipase and uptake of chylomicron triacylglycerol and cholestorol by perfused rat mammary gland. Biochim. Biophys. Acta. In press.

Linder, C., Chernick, S.S., Fleck, T.R.C. and Scow, R.O.: Lipoprotein lipase and uptake of chylomicron triglyceride by skeletal muscle of rats. Am. J. Physiol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM 15401-04-LNE

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Transport of lipids, hormones and enzymes in tissues, cells and membranes

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

PI: Robert O. Scow, Chief, Section on Endocrinology, LNE, NIAMDD
E. Joan Blanchette-Mackie, Research Biologist, LNE, NIAMDD

OTHER: Angelo A. Ucci, Jr., Research Associate, LNE, NIAMDD

COOPERATING UNITS (if any)
Z01-15400-02

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Endocrinology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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

The main objectives of the project are: (1) Determine structure and metabolism of cells that synthesize and secrete lipids and hormones. (2) Determine mechanism of transport of lipids, hormones and enzymes across capillary endothelium, basement membrane, and extracellular space in different tissues. (3) Determine mechanism of uptake of lipids, hormones and enzymes by different kinds of cells. (4) Study structure and role of cell membranes in lipid metabolism. (5) Study interaction of lipoprotein lipase with chylomicrons in vitro and in capillaries.

PHS-6040
(12-75)

Project Description:

Objectives: To study the structure and metabolism of cells that synthesize and secrete lipids and hormones. To study the transport of lipids, hormones and enzymes across the capillary endothelium, basement membrane and extracellular space in different kinds of tissues. To study the uptake of lipids, hormones and enzymes by different kinds of cells. To study the structure of cellular membranes and their role in lipid metabolism. To correlate structural with biochemical changes induced experimentally in cells and tissues. To study the effect of lipoprotein lipase and different phospholipases on the composition and structure of chylomicrons in vitro.

Methods Employed: Animals of different species and different ages and in various physiological states (e.g. fasting, pregnant, lactating) are used to study with biochemical and morphological techniques the synthesis and secretion of lipoprotein triacylglycerol by liver and intestines, and utilization of plasma triacylglycerol by adipose tissue, mammary gland, crop sac (pigeon) and muscle. Perfused adipose and mammary tissues are used to locate with cytochemical techniques the sites of action of lipoprotein lipase, the mode of transport of glycerides, fatty acids and cholesterol between blood and tissue cells. Perfused tissues are also used to determine the immunocytochemical 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 changes induced when the triacylglycerol core is reduced by lipolysis.

Major Findings: Effects of lipoprotein lipase, phospholipase A_2 and phospholipase C on chylomicrons in vitro. Lipoprotein lipase purified from bovine milk readily hydrolyzed triacylglycerol in chylomicrons stepwise to glycerol and fatty acids if sufficient albumin was present in the medium to bind the fatty acids produced. When lipolysis occurred at maximal rate, however, there was a transient accumulation in chylomicrons of monoacylglycerol, up to 1/3 of that formed, before it was hydrolyzed to glycerol and fatty acids. This delay in hydrolysis probably reflects both positional specificity of lipoprotein lipase for primary ester bonds of acylglycerols and time needed for nonenzymatic isomerization of 2-monoacylglycerol to 1(3)-monoacylglycerol, which can be hydrolyzed by the enzyme to glycerol and fatty acids.

Purified lipoprotein lipase also hydrolyzed phosphatidylcholine of chylomicrons to 2-acyl lysophosphatidylcholine and fatty acid. The rate of hydrolysis, which was always less than 5% of that for triacylglycerol, was increased with enzyme concentration and decreased when fatty acid binding sites on albumin were limited in the incubation medium. Others have proposed that hepatic triacylglycerol lipase is principally responsible for phospholipase A_1 activity in postheparin plasma. Our findings indicate that lipoprotein lipase can account for some of the phospholipase activity in postheparin plasma.

Phospholipase A_2 and phospholipase C both hydrolyzed chylomicron phosphatidylcholine, >92% in 10 min, but not triacylglycerol. The resultant phosphatidylcholine-deficient chylomicrons, which could be concentrated by ultracentrifugation and resuspended in incubation medium, were readily depleted of triacylglycerol when incubated with lipoprotein lipase in medium containing sufficient albumin to bind fatty acids formed. Electron microscopic analyses showed that both types of phosphatidylcholine-deficient chylomicrons resembled control chylomicrons in that they were spherical with smooth surfaces.

Water spaces lined by lipolytic products developed in intact chylomicrons when incubated with lipoprotein lipase in medium containing limited albumin. Water spaces also developed, under the same conditions, in chylomicrons pretreated with phospholipase C, but not in those pretreated with phospholipase A_2. Laminar structures were present, however, in the incubation medium of the latter group. Chylomicron remnants, seen as flat sacs by negative staining, were produced when intact chylomicrons or phosphatidylcholine-deficient chylomicrons of either type were incubated with lipoprotein lipase in medium containing excess albumin. These findings indicate that phosphatidylcholine, which accounts for about two-thirds of the lipid in the surface film, can be removed from the film without disrupting the chylomicrons or blocking the action of lipoprotein lipase on triacylglycerol in the core.

A model for lipid transport by lateral diffusion in cell membranes. In vitro studies reported last year showed that products of lipolysis (monoacylglycerol and fatty acids) are retained in chylomicrons incubated with purified lipoprotein lipase when fatty acid acceptors are limited in the medium. Morphological studies of these chylomicrons showed that the lipolytic products accumulate in interfacial planes between core triacylglycerol and water, and that the chylomicron surface film is extended as a lipid monolayer lining and spiralling within aqueous spaces that form in the chylomicrons. These results indicated that lipolytic products can move by lateral diffusion in the monolayer, and that lipolysis will continue as long as the monolayer can expand.

Perfusion studies in mammary and adipose tissue showed that uptake of chylomicron triacylglycerol from blood involves hydrolysis of triacylglycerol by lipoprotein lipase to partial glycerides and fatty acids which cross the capillary endothelium to be utilized by the tissue cells. Morphological studies showed that chylomicrons become attached to the capillary endothelium during uptake of triacylglycerol by the tissue, that the luminal surface of the endothelium is connected with the basal surface by membranes lining channels (vesicles and vacuoles) which cross the endothelial cells, and that there are sites of apposition between different cells in tissues. These findings suggested that products of lipolysis within capillaries might move across the endothelium and between cells by lateral diffusion in cell membranes. Therefore the possibility was considered that fusion of the external leaflets of plasma membrane of apposing cells might form a continuous lipid interfacial plane in which lipolytic products could move.

The hypothesis that lipolytic products can locate and move by lateral diffusion in cell membranes is supported by the following observations:

(1) Hepatic cells of fed rats contained lipid inclusions which consisted mostly of triacylglycerol with stacks of bilayered lamellae at the periphery. The lamellae, which sometimes extended into the osmiophilic core of the inclusions, were structurally associated also with endoplasmic reticulum. The electron-opaque areas within the inclusions were replaced with electron-lucent areas when liver fixed with glutaraldehyde was incubated at 37°C. These findings suggest that the electron-lucent areas result from enzymatic hydrolysis of triacylglycerol with accumulation and diffusion of the amphiphilic lipid products within the lamellae and endoplasmic reticulum.

(2) Mammary tissue of lactating rats contained bilayered lamellae that extended from the endothelial plasmalemma toward the capillary lumen, sometimes in close association with chylomicrons or other lipid particles in blood. Lamellae were also seen in the intercellular space, between capillary endothelium and mammary epithelial cells. In some sections, the intercellular lamellae appeared to be spiralled extensions of plasmalemma of endothelial and parenchymal cells. Lamellae were also present within alveolar cells, spanning the space between the plasmalemma and the bilayered membranes of rough surfaced endoplasmic reticulum, the site of esterification of fatty acids for secretion in milk. Lamellae were sometimes associated with milk lipid droplets near the basal plasma membrane of the epithelial cells.

(3) Prolactin-stimulated crop sac. Electron microscopic study of the crop sac of pigeons treated with prolactin showed that capillary endothelial cells and basal epithelial cells had microvilli-like cytoplasmic protrusions which penetrated their respective basement membranes and sometimes opposed each other in the intercellular space. When the crop sacs were fixed with glutaraldehyde at 24°C, the cytoplasmic projections described above often bore lamellar whorls, and lamellae were seen along the lateral borders of basal epithelial cells as well as on the surface of lipid droplets within crop epithelial cells. However, when the tissue was fixed with glutaraldehyde at 0-4°C the number of such lamellae seen was markedly reduced and none were found in tissues fixed first in 2% osmium tetroxide at 0-4°C.

The lamellar structures seen in mammary gland and crop sac probably result from hydrolysis of blood triacylglycerol during and after fixation of tissue with glutaraldehyde, especially at room temperature. It is likely that the amphiphilic lipids formed, partial glycerides and fatty acids, diffuse in the outer leaflet of the plasmalemma of endothelial cells, along cytoplasmic protrusions, to the outer leaflet at the plasmalemma of parenchymal cells. Also, the amphiphilic products may accumulate in the outer leaflet of a cell membrane and extend outwardly as bilayered lamellar whorls, since re-esterification in these tissues is inhibited by glutaraldehyde. The findings indicate that there is a fusion of the external leaflets of plasmalemma of opposing cells which forms a continuous water-lipid interfacial plane in which lipolytic products can move by lateral diffusion.

Significance to Biomedical Research and the Program of the Institute. Lipoprotein lipase is necessary for the uptake of chylomicron triacylglycerol from blood by extrahepatic tissues. Other studies in this laboratory showed that the enzyme acts in capillary endothelium to hydrolyze triacylglycerol to partial glycerides and fatty acids which cross the capillary to be utilized by tissue cells. Recent studies showed that uptake of chylomicron cholesterol by extrahepatic tissues is also dependent on lipoprotein lipase activity. We have proposed a model, based in part on the above findings, for transport of partial glycerides, fatty acids and cholesterol from chylomicrons to cells in tissue by lateral diffusion in a continuous water-lipid interfacial plane consisting of the chylomicron surface film and the external leaflets of plasma and intracellular membranes of endothelial, interstitial and parenchymal cells. (See Project Z01-AM 15400-02-LNE) It is also proposed that lipids derived from chylomicrons and other sources leave the interfacial continuum in parenchymal cells where they are esterified, to either triacylglycerol or cholesterol ester, and separate from the lipid interface to accumulate between the external and cytoplasmic leaflets of endoplasmic reticuluum. It is likely that this mechanism for transport of lipid in extrahepatic tissues operates in the wall of the large blood vessels. We think that FFA mobilized from adipose tissue as well as amphiphilic proteins and hormones may also move between blood and parenchymal cells by lateral diffusion in the above interfacial continuum. These possibilities are under study.

Proposed Course. We plan to use the freeze etching technique, combined with electron microscopy, to study the transport of lipid from blood to tissue cells, the secretion of lipid by cells (intestinal, liver, mammary gland), and the interaction of lipoprotein lipase with chylomicrons. Immunocytochemical techniques will be developed for locating lipoprotein lipase in parenchymal cells and capillary endothelium and on chylomicrons incubated with the enzyme.

Publications:

Blanchette-Mackie, E.J. and Scow, R.O.: Retention of lipolytic products in chylomicrons incubated with lipoprotein lipase: Electron microscope study. J. Lipid Res. 17: 57-67, 1976.

Blanchette-Mackie, E.J. and Scow, R.O.: Scanning electron microscopic study of chylomicrons incubated with lipoprotein lipase. Anat. Rec. 184: 599-610, 1976.

Scow, R.O., Blanchette-Mackie, E.J. and Smith, L.C.: Role of capillary endothelium in the clearance of chylomicrons: A model for lipid transport from blood by lateral diffusion in cell membranes. Circ. Res. In press.

Scow, R.O. and Egelrud, T.: Hydrolysis of chylomicron phosphatidylcholine in vitro by lipoprotein lipase, phospholipase A2 and phospholipase C. Biochim. Biophys. Acta. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM 15402-02-LNE

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Pharyngeal lipase and digestion of dietary fat

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

PI: Robert O. Scow, Chief, Section on Endocrinology, LNE, NIAMDD
Sidney S. Chernick, Scientist Director, LNE, NIAMDD

COOPERATING UNITS (If any)
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NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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

Earlier studies from this laboratory showed that tissues in or near the pharynx secrete in man a lipase that acts in the stomach, at pH 5.4, to hydrolyze triacylglycerol to di- and monoacylglycerols and fatty acids, and that this is the first step in the digestion of dietary fat. The products formed are amphiphilic and, thereby facilitate emulsification of triacylglycerol before it leaves the stomach.

The objectives of this project are (1) isolate and purify pharyngeal lipase from tissues of rats and other species, and from pharyngeal secretions of man. (2) Characterize the action of pharyngeal lipase. (3) Study the regulation and mode of secretion of pharyngeal lipase. (4) Determine role of pharyngeal lipase in fat digestion in normal and diseased states, in both infants and adults.

Project Description:

Objectives: To purify and characterize pharyngeal lipase isolated from tissues of rats and other species, and from pharyngeal secretions of man. To study the regulation and mode of secretion of the lipase. To determine the role of pharyngeal lipase in the digestion of dietary fat in man and other species.

Methods Employed: Pharyngeal tissues from rats and calves, and pharyngeal secretions of man will be used as sources of pharyngeal lipase. Techniques used elsewhere for isolation and purification of pancreatic lipase will be modified as necessary for extracting the enzyme from tissues and secretions. Collaborative studies will be continued at the Department of Pediatrics, Oulu, Finland to determine the role and regulation of pharyngeal lipase activity in neonatal and premature infants.

Major Findings: Earlier studies from this laboratory showed that tissues in or near the pharynx in man secrete a lipase that acts in the stomach, at pH 5.4, to hydrolyze triacylglycerol to di- and monoacylglycerols and fatty acids, and that this is the first step in the digestion of dietary fat. A similar activity was found, in fluid collected from the proximal end of the esophageal fistula of an adult patient on the NIAMDD-DD service. About 15 liters of thick fluid were collected over a period of 2 weeks and stored frozen. Mucus in the fluid was liquified by sonication. The lipolytic activity in the fluid is remarkably stable since only 30% was lost when stored for 4 months at 4°C, and none was lost when stored in the frozen state. Purification of the lipase in this fluid is now under way.

Recent collaborative studies at the Department of Pediatrics, University of Oulu, Finland indicates that lipolytic activity is present in the stomach contents of both premature and neonatal infants, and the pH curve of the activity suggests that the activity is due, at least in part, to pharyngeal lipase. This suggestion is supported by the finding of a similar enzyme activity in aspirates from the upper esophagus of a child with esophageal atresia, and in aspirates from the stomach of a child with pyloric stenosis, in which the pancreas and duodenum can be excluded as a source of lipolytic activity in the stomach. It is of interest that lipolytic activity was found in stomach contents of premature infants with gestational age as young as 33 weeks. Studies are in progress to determine the importance of pharyngeal lipase in digestion in infants.

Significance to Biomedical Research and the Program of the Institute: It is now widely accepted that pharyngeal lipase accounts for the lipolytic activity present in gastric contents and that the products formed, especially monoacylglycerol and fatty acids, facilitate emulsification of triacylglycerol before it leaves the stomach. The importance of this enzymic reaction in patients with abnormal fat digestion is not yet known.

Proposed course: Studies will be continued to determine the role of pharyngeal lipase in fat digestion and to determine what factors regulate its synthesis and secretion. Studies will also be made to determine the site of synthesis and secretion of pharyngeal lipase in man. Isolation and purification of the lipase from tissues of rats and from secretions of man will be continued.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 15500-16 LNE

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Large-scale Processing of Biological Materials.

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

PI: Mr. D. L. Rogerson, Jr. Head, Pilot Plant LNE NIAMDD
OTHER: None

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Nurition and Endocrinology

SECTION
Office of Chief

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland, 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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

Production of large quantities of microorganisms for the isolation and purification of biologically imporant macromolecules and cell particulates and to assist NIH investigators in the scale-up and processing of plant and animal tissue for biological substance such as enzymes, vitamins, alkaloids, etc.

PHS-6040
(12-75)

Project Description

Objectives: To provide facilities and services for the processing and/or production of large amounts of natural materials, such as bacteria, animal tissues, cells, blood, urine and plant materials, so that they may be studied under standard laboratory conditions.

Methods Employed: To satisfy the large-scale production needs of NIH investigators, 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 300-liter and one 1100-liter fermentors are utilized for growing microorganisms under a variety of conditions. Bacterial cells and yeasts are harvested by means of laboratory or large-scale AS-16P and AS-26P 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 using conventional fractionation procedures and the newer chromatographic techniques.

Major Findings: During the past year, a total of 260 requests were processed for investigators: NIAMDD 122; BoB 42; NICHD 32; NCI 16; NHLBI 13; NIMH 8; NIDR 7; NINCDS 7; NIAID 3; NEI 1; and others 8. 184.5 kg quantities (42,590 liters of cultures) of 18 organisms were produced. The microorganisms grown during this period include wild type and mutant strains of *Arthrobacter luteus, Bacillus amyloliquefaciens, Bacillus pumilis, Bacillus stearothermophilus, Bacillus subtilis, Bordetella pertussis, Clostridium pasteurianum, Escherichia coli, Euglena gracilis, Haemophilus influenzae, Haemophilus parainfluenzae, Klebsiella cloaceae, Neisseria meningitidis, Pneumococcus sp., Pseudomonas sp., Saccharomyces cerevisiae, Salmonella typhimurium, Streptococcus mutans.* Some of the organisms were used for the production of bacteriophages MS2, lambda, T_4 and T7, others were used to produce extracellular enzymes. Microorganisms were grown 68 times in the two 10-liter, 70 times in the 50-liter, 62 times in the 300-liter and 18 times in the 1100-liter fermentors. Of the 200 fermentations, 23 (11.5%) were contaminated with 19 of these traced to the inocula supplied by the requesting investigator.

Studies were continued with the Division of Bacterial Products, Bureau of Biologics for polysaccharide production by *H. influenzae* and isolation of the enterotoxin proteins from special strains of *E. coli*. Spheroplasts were produced for the NHLBI from *E. coli* using 10-, 50-, and 300-liter fermentors in series. Some of the spheroplasts were recovered in the new Electro-Nucleonics RK zonal centrifuge in an attempt to improve the quality of the product. The Gaulin laboratory homogenizer was used 45 times to rupture cell suspensions of yeast, protozoa or bacterial cells.

Large scale processing activities consisted of the isolation of polynucleotide phosphorylase from 1 kg (dry) *Micrococcus lysodeikticus* cells for NCI; the preparation of Exonuclease-5 from 3.6 kg *E. coli*, ATPase from a 20-liter

culture of *Acanthamoeba castellanii* and preliminary isolation studies for the extraction of enzymes from chicken livers using the new RK centrifuge for NHLBI; the fractionation of 18.6 liters equine meningococcal group B anti-serum and preparation of IgG component from 18.3 liters of burro cholera toxin antiserum for the BoB; the isolation from two 1100-liter cultures of *B. amyloliquefaciens* the enzyme, Barnase, and its inhibitor, Barstar, from 37 kg of cells and the preparation of three two-gallon quantities of hydroxylapatite for NIAMDD. Various plant and animal tissues were either dried, and/or ground in the large ovens, mills and related equipment and solutions were centrifuged such as 30 liters of Killam rat virus and several 15-liter cultures of special *E. coli* mutants. Large volumes of supernatants were concentrated including the lyophilization of 9 liters to dryness in the Stokes freeze-dryer, 8 liters to 700 ml in the circulating evaporator and 1100 liters to 25 liters in the Pfaudler reactor.

Publications:

Rogerson, D. L. and Rushizky, G. W.: An improved method for the preparation of MS-2 bacteriophage in large quantities. Anal. Biochem. 67, 675-678, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15600-21 LNE

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies in Experimental Nutrition.

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

PI:	E. G. McDaniel	HS Director	LNE NIAMDD
OTHER:	G. Laqueur	Chief, LEP	LEP NIAMDD
	J. C. Smith	Chief, Trace Ele. Lab.	VA Hospital
	R. S. Yamamoto	Research Chemist	CMT NCI

COOPERATING UNITS (if any)

VA Hospital, Washington, D. C. (Trace Element Lab.)
Carcinogen, Metabolism and Toxicology Laboratory, NCI

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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

It has been shown that two precursors of the aglycone of cycasin (dimethyl hydrazine and azoxymethane) are carcinogenic in germfree as well as in non-germfree rats. The location of the tumors observed with these two compounds differ from those produced by the aglycone itself, indicating that the two compounds themselves may be active, or can be converted to active compounds other than the aglycone by the animal tissue enzymes.

It has been observed that maintenance of normal levels of plasma vitamin A in addition to being dependent upon adequate amounts of zinc, is also related to growth of the animal and/or dietary intake. It was further observed that lack of growth also resulted in below normal levels of plasma zinc. It has been possible to maintain normal levels of plasma zinc in the absence of growth by intraperitoneal administration of rather large daily doses of zinc. Preliminary results indicate that the plasma vitamin A may be increased even in the absence of growth when the plasma zinc is thus maintained.

Project Description

Objectives: With Drs. Laqueur and Yamamoto we have continued to study the actions of cycasin and related compounds with respect to the production of tumors in rats, and the possible influence of intestinal bacteria upon these actions. It was previously shown that cycasin is non-toxic in adult germfree rats, although the compound routinely produces tumors in conventional rats, previously germfree rats which have been infected with certain types of bacteria, ex-germfree rats which have been permitted to develop a supposedly "Full Flora", and in infant germfree rats. Previously germfree rats infected with specific strains of bacteria known to be unable to convert cycasin continued to behave as germfree rats and exhibited no toxic reaction to the compound. Animals infected with specific types of bacteria which were known to be able to convert cycasin then behaved as conventional animals and responded to the toxic effects. Although the very young germfree rat is able to convert cycasin to the toxic form this ability is lost after the first few weeks in the life of the rat. It has also been shown that the aglycone of cycasin (MAM), a compound produced from cycasin by the bacteria produced tumors in germfree as well as in non-germfree rats. Experiments are in progress to determine which intermediate compounds in the conversion of cycasin to its aglycone are the substances actually responsible for the carcinogenic actions. Certain precursors in the in vitro synthesis of the aglycone of cycasin, which have been shown to be carinogenic in conventional rats, are being examined. Two of the compounds at present under study dimethyl hydrazine (DMH) and azoxymethane (AOM) were found to be equally effective in producing tumors in both germfree and non-germfree rats, thus indicating that the compounds themselves are active, or that if further conversion is necessary it is accomplished by the animal's own enzymes. Azoxymethane appeared to be the more toxic of the two and resulted in death due to acute toxicity in a number of cases. Tumors have been observed which develop over the period of a year following administration of the drugs. Severity of the action is increased in relation to the number of doses administered. Tumors of various sizes were observed in most of the animals treated, primarily in the intestine, but also in other sites, especially the kidneys and liver. In many cases multiple tumors were observed which appeared to have developed independently of one another. Since the sites of tumors observed with these two compounds appear to differ from those seen when the aglycone itself was administered, it is suggested that the DMH and the AOM may themselves be active, or do not necessarily need to be converted completely to the aglycone. It is hoped to identify which intermediate compounds in the in vivo conversion of cycasin to it's aglycone are carcinogenic and which may be formed by the animal's own enzymes. It is further hopedto identify specific bacteria responsible for those compounds which occur only due to the action of bacteria.

With Dr. Smith experiments have been continued to study the interrelationships of vitamin A and the trace element zinc, and to attempt to determine the way in which zinc influences the mobilization and the transport and utilization of vitamin A, and the way in which metabolism of both of these factors appear to be influenced by the presence or absence of a state of growth of the animal. In a state of zinc deficiency the rat is readily able to accept and store normal amounts of vitamin A in the liver, but is unable to maintain normal levels of

the vitamin in the plasma. During the zinc deficient state there occurs also cessation of growth and abnormally low amounts of zinc in the plasma. Administration of zinc in addition to restoring normal growth and increasing plasma zinc to normal levels, also elevates the plasma vitamin A to normal amounts even though no vitamin A is administered. This increased plasma A must therefore have been mobilized from the liver stores. Still another variable which becomes a factor following zinc therapy is the increased food intake. It is of interest to determine in what way these various factors may be interrelated in the influence observed on the apparent mobilization and transport of vitamin A. It has been observed that if growth is prevented by restricting food intake during zinc therapy the plasma vitamin A is not fully restored to normal levels. It is further observed that the plasma zinc remains lower than normal when growth is prevented. This poses the question as to whether the low plasma is the direct result of lack of growth, or indirectly due to the low plasma zinc resulting from the growth restriction. It was shown however that even though plasma A remained low following zinc therapy with growth restriction, the retinol-binding protein (RBP) in the liver was increased by the zinc. Plasma levels of RBP, vitamin A and zinc were all restored to normal when growth was permitted by lifting the food restriction. This observation may suggest that although zinc permitted synthesis of RBP and made possible the mobilization of liver vitamin A, factors associated with growth may have been necessary in transporting it into the plasma. In order to determine if the low plasma A associated with growth restriction was due to the low plasma zinc, we have been able to maintain normal plasma zinc levels during growth restriction by administering intraperitoneally rather large doses of zinc daily. Preliminary results were promising in that higher plasma A values were observed in some animals.

It is interesting that it appears to be quite necessary for young animals (less than adult) to be growing in order to maintain normal levels of vitamin A and zinc in the plasma, while the adult animal which has ceased growing apparently does not experience this difficulty. To determine if this was due to some physiological change with age rats were maintained on diets sufficiently low in protein to restrict growth to the degree that would cause abnormally low levels of both plasma vitamin A and zinc in young animals. There was no apparent increase with age in the ability to maintain higher levels of the two factors. However increases were observed when growth was again permitted. Experiments are planned using adult rats to determine if the normal plasma levels of vitamin A and zinc may be more closely associated with food intake than with actual growth. In the young growing rat it is impossible to dissociate food intake and growth.

We have been interested in the crippling which is observed in vitamin A deficiency and which appears much earlier and more severely in animals which have been subjected to simultaneous deficiencies of zinc and vitamin A. The crippling occurs following treatment with zinc alone,and is prevented by treatment with both zinc and vitamin A. The symptoms of vitamin A deficiency occur earlier and are more severe following a period of rapid growth caused by the zinc therapy. We have attempted to increase the severity of the crippling and thereby make any accompanying lesions more marked by maintaining the animal in a crippled state for long periods. This was done by treating with vitamin A

at infrequent intervals. Pathological examination of these animals is being done at the V.A. Hopsital. To date no abnormalities in bone formation have been noted, but there are indications of possible abnormalities in some nerve and muscle tissues. Comparisons are being made of various other tissues of animals subjected to deficiencies of vitamin A and zinc individually and of the double deficiency. To date the typical eye lesions observed in vitamin A deficiency have not been seen in the zinc deficient or doubly deficient animals. We will attempt to maintain animals in a state of zinc deficiency for longer periods. Since zinc deficiency occurs much more rapidly than does vitamin A deficiency it is possible that the zinc deficient animal does not normally survive sufficiently long to have developed the lesion.

Publications:

Smith, J. C., Brown, E. D., McDaniel, E. G. and Chan, Winnie: Alterations in Vitamin A Metabolism during Zinc Deficiency and Food and Growth Restriction. J. Nut. 106, 569, 1976.

ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMISTRY AND METABOLISM

A. The Role of Hepatic Plasma Membranes in the Recognition and Catabolism of Serum Glycoproteins. Continuing investigation into the mechanism whereby hepatic membranes find and transport glycoproteins into the parenchymal cells may be summarized as follows:

1. The purified binding protein isolated from rabbit liver has been shown to be a glycoprotein whose binding activity is destroyed by exposure to neuraminidase. Further information on the nature of the inactivation process was sought by examination of the intrinsic carbohydrate moiety. Two widely different glycopeptides were isolated and their sequence determined. The larger, acidic glycopeptide contained 25 monosaccharide units attached to Asparagine; the smaller, neutral glycopeptide contained 12 monosaccharide units. The disposition of the two classes of glycopeptides on the two non-identical subunits of the hepatic binding protein has been determined.

2. A methodology has been developed for the detection and quantitation of desialylated glycoproteins in serum and evidence has been obtained to document increased levels of circulating asialoglycoproteins in patients with impared hepatic function (cirrhosis and hepatitis). These studies have revealed abnormally high levels in avian and retilian species concomitant with the absence of the specific galactose recognizing hepatic receptor. The evolutionary significance of this finding, has been furthered by the detection of a new avian binding protein which recognizes and binds terminal N-acetylglucosamine residues.

3. The above binding protein has been isolated in homogeneous form free chicken liver and has been characterized as a glycoprotein with properties analogous to, but distinct from, the galactose specific protein of mammalian liver.

4. Studies on the intracellular localization of the mammalian binding protein in rat livers has demonstrated it to be present in large amounts in the Golgi, smooth microsomes, plasma membranes and lysosomes. Lesser amounts have also been identified in the mitochondria, peroxisomes, and nuclei. The role of this protein in intracellular recognition is being investigated.

B. The Formation of the Yeast Primary Septum. Work on this topic has continued in three different areas, i.e. localization and solubilization of the chitin synthetase zymogen, purification of the chitin synthetase activating factor, localization of chitin in the cell wall and bud scars and search for mutants in the chitin synthetase system.

1. Localization and Solubilization of Chitin Synthetase Zymogen. As mentioned in the previous report, the successful preparation of intact plasma membranes permitted to establish that the chitin synthetase zymogen is attached to the membranes. It appears that the enzyme is facing the interior of the cell, because it can be irreversibly inactivated by glutaraldehyde only after yeast protoplasts are lysed and the inner side of the membrane is exposed.

An ultrastructural study of the membranes by freeze-fracture electron microscopy has begun. Preliminary results show that the isolated membranes are similar in structure and particle distribution to those seen in intact protoplasts and intact cells.

After several attempts with a number of detergents, the chitin synthetase zymogen was finally "solubilized" from the membranes with very good yield by the use of 1% digitonin. "Solubilization" is defined here as lack of sedimentability at 200,000 x g for 1 hour. It was gratifying that the solubilized enzyme was still in the zymogen state, i.e. it only showed activity after treatment with trypsin or yeast protease. This result strongly supports the idea that activation of the enzyme is due to a precursor-active form relationship, rather than to an artifactual phenomenon, such as would be the opening by proteolytic action of a vesicle containing chitin synthetase in its inner face. The solubilized enzyme produces insoluble chitin without any added primer. Work is now in progress on the purification of the enzyme, which still appears to consist of a large aggregate.

2. Purification of the Chitin Synthetase Activating Factor. As described in the previous report, the yeast protease that converts the chitin synthetase zymogen into its active form has been purified by affinity chromatography on a sepharose column to which the proteinaceous inhibitor of the enzyme had been attached. The purified preparation had a very unusual behavior, in that it yielded no stainable band in conventional disc gel electrophoresis; however, when the electrophoresis was carried out in 4 M urea at acidic pH a single band could be observed. At the same pH, but in the absence of urea a much fainter band was observed, which coincided with proteolytic activity. It was concluded that the purified enzyme is homogeneous under these conditions. The ratio between proteolytic activity and ability to activate the chitin synthetase zymogen was constant throughout purification. A molecular weight of 55,000 was determined for the enzyme by gel filtration and sedimentation in a sucrose gradient. By titration of the activity it was established that the enzyme combines mole to mole with either its proteinaceous inhibitor from yeast or with phenylmethylsulfonyl fluoride. The latter result indicates that the protease contains one active serine group per molecule. The enzyme is also inhibited by very small concentrations of the actinomycete metabolites chymostatin and antipain. A comparison of its specificity with that of trypsin, using a series of synthetic substrates, showed a rather similar general pattern, with some clear differences.

3. Localization of Chitin in the Cell Wall and Bud Scars. Previous work has supported the notion that chitin forms the primary septum of yeast and that it remains embedded in the bud scars after cell separation. Nevertheless, we still lack a specific method to study the topology of chitin at the level of resolution of the electron microscope, in order to follow the formation of the septum in detail. Since wheat germ agglutinin has a very strong and specific affinity for chitin, it was thought that this lectin would make a good marker for the polysaccharide, after being coupled to ferritin, in order to make it visible under the electron microscope. Therefore, the agglutinin was purified to homogeneity from wheat germ extracts and attached to ferritin by the use of glutaraldehyde. Preliminary results indicate that the ferritin-bound lectin binds to chitin and can be visualized under the electron microscope. An unexpected and troublesome finding was that the agglutinin also binds to yeast glucan thus interfering with the observation. It appears, however, that this unspecific binding can be prevented by the use of chitin oligosaccharides at concentrations too low to interfere with the much stronger binding to chitin.

4. Search for Mutants in the Chitin Synthetase System. Previous studies with the chitin synthetase inhibitor polyoxin D had indicated that a temperature-sensitive mutant defective in the chitin synthetase system probably would yield lysed cell pairs at the non-permissive temperature. With this idea in mind and using the recently developed method for the screening of lysing mutant, a search for such mutant was continued. Although several strains with the above-mentioned phenotype were found, sofar none of them seems to be affected in chitin synthesis. The search continues along the same lines.

Biosynthesis of a Yeast Glucan. During investigations on yeast glycogen synthetase and its regulation, it was found that some radioactivity from labeled UDP-glucose was incorporated in a trichloroacetic-insoluble fraction. This material was not glycogen, because it was resistant to α and β-amylases. On the basis of its resistance to periodate, of the series of oligosaccharides produced by partial acid hydrolysis and of its degradation by a purified β-glucanase, it was identified in a preliminary way as a β-1,3-linked glucan. Since such glucans are major components of the yeast cell wall and appear to impart shape to the yeast cell, it was considered of importance to continue its study. Recently, particulate preparations have been obtained from protoplasts which can convert about one-third of the substrate into product in 20 min, with the use of about 2.0 mg of protein at a 5 mM concentration of UDP-glucose. The activity requires the presence of glycerol and of unknown factor(s) present in boiled yeast extracts. These factors can be substituted in part by the addition of ATP and magnesium ions. Because of these unusual requirements and of the importance of glucan for the structure of the yeast cell wall, the study of this enzymatic system will be actively pursued.

C. Function of Membrane Components in Bacteriocin Tolerance. The goals of this project were to purify the tolG protein, a major component of the outer membrane of E. coli, in sufficient quantities to permit chemical and biological characterization and to use this material to reconstitute bacteriocin sensitivity in bacteriocin-tolerant mutants. The major findings are:

1. The purified tolG protein is apparently identical with protein II*, purified by Henning and coworkers and protein B, purified by Bragg and coworkers. The following findings apply equally to all three preparations.

The tolG protein has an unusual property in that its mobility on polyacrylamide gels containing SDS depends upon how the protein was heated prior to applying to the gel. The apparent molecular weight is about 28,500 when heated at 50° whereas this value is increased to 33,500 when heated at 100°. The amino acid composition is not remarkable, the N-terminal amino acid is alanine and the purified material contains 2 moles of carbohydrate and no detectable lipid or phosphate. The purified material has no detectable biological activity even when the SDS is removed. Evidently the protein was irreversibly denatured by the SDS treatment.

2. Up to 70% of the cell membrane proteins can be incorporated into unilamellar phospholipid membrane vesicles. Mixing these vesicles with intact cells results in an association of the added membrane proteins with the cells. This finding, although preliminary, augers well for the incorporation of exogenous (purified) membrane proteins into intact cells. Similar unilameller phospholipid vesicles have been shown to fuse with the outer membrane of E. coli cells.

3. The tolF locus was shown to be phenotypically and genetically identical to the cmlB locus. tolF strains, like tolG strains, have been found to be missing a major outer membrane protein. The protein, missing in the two strains are distinct.

4. Cells are tolerant to colicin L-JF246. The most prominent alteration associated the mutation to tolerance is the loss of the tolG protein from the outer membrane. Tolerance in tolG cells can be overcome by a variety of techniques, each of which has the common feature that the integrity of the outer membrane is perturbed. These results suggest that there may be a direct interaction of colicin L-JF246 with the cytoplasmic membrane and the function of the tolG protein may be to assist the colicin molecule, or a portion of it, through the outer membrane barrier.

D. Hormone-Dependant Differentiation of Mammary Gland. The study of the molecular and cytological phenomena involved in development of the mammary gland attempts to understand the mechanism of insulin, prolactin, placental lactogen, glucocorticoids, estrogen and progesterone.

1. It was reported last year that super-active forms of insulin, prolactin and placental lactogen can be released from the corresponding sepharose complexes by treatment with proteins such as bovine serum albumin. It has since been observed that similar super-active hormones are also released when the nucleophile is NH_3. In this instance, monosubstituted guanidines (guanidinated hormones) are formed. The identity of the amino acid residue of the hormones which adds to the activated sepharose, leading ultimately to the formation of the soluble, super-active hormones, has not been determined. The small fraction of the total bound protein which gives rise, during treatment with NH_3, to hormones with enhanced biological material is not bound via lysine, since the released materials contain no detectable homoarginine.

2. The epithelium in the mannary gland of the adult made Swiss NIH mouse resembles the anlage in the embryonic female. We have used the male animal as a convenient source, then, of embryonic-like mammary epithelium. It has been observed that appropriate hormone treatment in vivo can elicit growth and differentiation of the male epithelium equivalent to that which occurs in the pregnant female. Such growth is not dependent upon insulin, but functional differentiation is dependent upon the hormone.

3. L-thyroxine (T_4) and L-Triiodothyronine (T_3) (but not the D-forms) evoke a 3-5-fold augmentation of α-lactalbumin activity induced in mammary explants in the presence of insulin, glucocorticoid and prolactin. Maximal effects are produced by 10^{-8} M T_4 and 10^{-10} M T_3. The accumulation of α-aminoisobutyric acid, glucose-6-phosphate dehydrogenase activity, galactosyl transferase activity and DNA synthesis are not affected by the thyroid hormones. The effect on α-lactalbumin appears to reflect an increased sensitivity of the cells to prolactin, but not to insulin or gluco-corticoid.

E. The Regulatory Function of Spermidine in Growth and Development of Mammalian Cells. Progress in this area may be summarized as follows:

In mammary epithelium, the concentration of spermidine increases markedly during lactation. A similar increase has been found during the hormonal induction of lactogenesis in vitro, which is effected by cultivation of mouse mammary explants with insulin, glucocorticoid and prolactin. Previous studies on the role of spermidine with this in vitro system have presented several lines of evidence indicating that the polyamine may serve a vital function in milk protein synthesis, possibly by mediating the effect of glucocorticoid.

Based on these previous findings, a series of studies to elucidate the mechanism of hormonal control of spermidine formation during mammary development in vitro has been undertaken Since the major biosynthetic pathway of spermidine in cultured mammary cells probably involves the following steps, i.e., arginine → ornithine → putrescine → spermidine, the effect of hormones on the enzymes which catalyze each of these steps has been examined.

Recent studies have shown that prolactin, in the presence of insulin, causes a several-fold increase in the activity of arginase, an enzyme which catalyzes the formation of ornithine. More recently, it has been found that mouse mammary epithelium possesses two forms of arginase, one being particulate-bound and the other in the soluble fraction. The stimulatory effect of prolactin was largely on the soluble form of the enzyme, and this enzyme appears to be involved in the polyamine biosynthesis.

The activity of ornithine decarboxylase, which converts ornithine to putrescine, increases biphasically with one peak being elicited simply by incubating the tissue in a synthetic medium and the second peak being stimulated by both insulin and prolactin. Several lines of evidence suggest the involvement of cyclic AMP as a stimulant of the first peak of activity.

F. Biochemical Lesions in Genetic Mucopolysaccharidoses. Studies undertaken on the hereditary biochemical defects in lysosome dysfunction have revealed:

1. Previous studies have indicated the existence of two forms of iduronidase, corrective and non-corrective for the Hurler syndrome in cell culture. By means of affinity chromatography, it has been possible to separate the two forms; only the corrective form, of higher molecular weight, is efficiently internalized by Hurler fibroblasts.

2. A new assay for iduronidase, based on the hydrolysis of tritium-labeled iduronosyl-anhydromammitol, has been applied to the purification of the enzyme and to the diagnosis of the Hurler defect.

G. Differentiation of Lymphoid Cells. The purpose of this project is to study the differentiation of the cells involved in the immune response with regard to all types, all interactions and control mechanisms. Major findings include the following:

Rabbit lymph or spleen cell populations cultured in vitro in the presence of fetal calf serum are induced to produce immunoglobulin M-secreting cells. The induction of such immunoglobulin production as well as DNA synthesis was inhibited when cells were cultured with sera from a variety of species despite the presence of fetal calf serum. An early event in the induction process is affected by serum because its addition at 36 hours was ineffectual and the presence of serum for only the first 24 hours yielded the same inhibition as the presence of serum throughout the tissue culture period.

The induction of immunoglobulin production and DNA synthesis were equally inhibited by the same range of serum concentrations. Unlike conventional inhibitors of DNA synthesis, the inhibitory sera exhibited selective specificity with regard to the kinds of cells that could be affected. From this and other data it has been demonstrated that bone marrow (B) cells rather than thymus-derived (T) cells or adherent cells, are the site of action of the inhibitor.

The sera of all species examined were inhibitory except for fetal sera. Ascites fluid and lymph node extracellular fluids contained less inhibitor than found in the serum of the same animal and lysates of washed lymph node cells were devoid of inhibitor. Although fetal bovine serum and new born bovine serum did not contain the inhibitor, it was detectable within 24 hours of parturition.

B-cells have been demonstrated to be the direct target of LPS-enhanced induction of immunoglobulin production, while T-cells have a regulatory role. Thus, following depletionof the T-cells of spleen with anti-thymocyte serum, the enhancement of immunoglobulin production of the treated cells by LPS was found to be dependent on the number of thymocytes added. Moreover, the prior incubation of T-cell-depleted spleen cells with LPS resulted in effective enhancement of immunoglobulin production when such T-cell-depleted spleen cells and thymocytes were combined after removal of LPS. On the other hand, an identical experiments, except that thymocytes instead of T-cell-depleted spleen cells received the prior incubation with LPS, did not result in enhancement of immunoglobulin production.

A relationship between the effect of LPS on immunoglobulin production and the effect of LPS on DNA synthesis was indicated by the findings as follows: (1) the optimal dose of LPS was the same for both activities, (2) the enhancement of immunoglobulin production by LPS was invariably accompanied by increased DNA synthesis, and (3) when DNA synthesis was inhibited, enhanced immunoglobulin production was inhibited. It should be noted that the obligate requirement for DNA synthesis is consistent with a proliferative function for LPS on B-cells.

H. Glutathione Transferases as Catalysts and Binding Proteins. The glutathione S-transferases are among the major detoxication systems of the animal kingdom and perform this function by related mechanisms which all involve binding of protein and ligand. Work with these enzymes has provided homogeneous transferases from both human and rat liver. The availability of clean systems has allowed extensive studies on the chemical and kinetic mechanisms of the enzymatic reaction as well as the specificity of binding.

Studies with these enzymes suggest that they are almost as avid as is albumin in both variety and tenacity with which they will bind ligands. Not only are naturally assuming compounds such as bilirubin and heme bound with dissociation constants of the order of 10^{-6} M, but a vast number of other lipophilic compounds serve as ligands. Current ideas of bilirubin excretion include the proposal that bilirubin is stored, while attached to the transferases intrahepatically but, upon glucuronidation, the conjugate is released at the cell surface and immediately transported through the bile coniculi. Our own results (with Allen Wolcoff) negate the hypothesis by demonstrating the presence of large quantities of conjugated bilirubin intrahepatically and by showing that the binding constants of the transferases for bilirubin conjugates are almost as high as those for bilirubin itself.

The catalytic reaction is also being studied with the conclusion that the transferases appear to be primitive enzymes. Because of their aridity in binding, they serve to passively hold compounds with highly electrophilic atoms in close proximity to glutathione. Their active function is in allowing glutathione to ionize at a lower than normal pH and thereby to become the highly reactive nucleophile, glutathione thiolate.

Work on glutathione conjugation by enzymes from liver is intimately related to a general mechanism of aromatic oxidation as well as to the biological problem of detoxication of noxious compounds. It is expected that investigations dealing with the above-noted projects will be continued and a study of other model reactions will be initiated.

I. Biochemical Abnormaity Underlying Human Cystinosis. The present project describes current attempts to define the biochemical basis of cystinosis, a rare and often fatal inherited disease of children characterized by the abnormal accumulation of cystine within lysosomes of certain cells of the body.

Several different cell lines from individuals with the lysosomal storage disease mucolipidosis II (I-cell disease) have been found to contain abnormally high intracellular levels of cystine (1-5 nanomoles/mg protein), approaching the levels usually encountered in cystinotic cells (5-15 nanomoles/mg protein). The identification of cystine in these I-cells was established by: (1) automated amino acid analysis; (2) appearance of a prominent radioactive peak corresponding in position to authentic cystine following high voltage electrophoresis of extracts of cells incubated in the presence of ^{35}S-cystine; (3) radioassay with the specific cystine-binding protein derived from E. coli. The intracellular component was further established as cystine, and not cysteine, by disruption of the cells in the presence of N-ethylmaleimide, a specific thiol-binding agent. Crude fractionation of extracts of I-cells with high intracellular cystine by differential centrifugation showed that, as in the case of cystinotic cells, a significant proportion of cystine was localized in the granular fraction. Several fibroblast cell lines from individuals with the related but less severe storage disease known as mucolipidosis III were examined and found to contain normal amounts of cystine.

J. Studies on Naturally Occurring Sulfur Nucleotides. The purpose of this project is to explore the two-enzyme trans-sulfur system which forms 4-thiouridine in tRNA and to determine the role of this nucleotide in the tRNA molecule.

1. A rough mapping of the location of the two enzymes in the 4-thiouridine sulfurtransferase system was carried out by examining the enzyme levels in a series of 44 F' strains of E. coli K12 carrying known segments of duplicated K12 chromosomal material in the episome. Gene dosage effects were observed in two regions, between pur E and lip for Factor A and between his and mglA for Factor C. Deletion mutants in these broad areas are being screened for lack of 4-thiouridine in their tRNA or for lack of the enzymes. In addition, phage P2-induced K12 deletions obtained from various sources are being "cured" of the P2-episome by acridine orange and also screened.

2. Factor A, the first enzyme in the synthesis of 4-thiouridine will bind to Poly U-agarose, but not to Poly A-agarose or agarose along. This correlates with the fact that Poly U, but not Poly A, can inhibit the thiolation of tRNA. Under specific salt conditions, the pH optimun for binding is 6.4. The method is useful for purification of the enzyme.

3. The tRNAs of plant pathogens which stimulate plant growth contain methylthioribosyl-zeatin, a cytokinin-active zeatin of the type previously reported only in higher plants. The nucleotide was found in the tRNA of Rhizobium leguminosarum, Agrobacterium tumefaciens and Corynebacterium fascians, all of which stimulate plant growth. On the other hand it was absent in Erwinia Amylovora, an organism which is pathogenic without stimulating growth.

K. Thermodynamic and Kinetic Studies of Protein Structure. At pH 8.5, pepsinogen is converted into a form which cannot be activated to pepsin at low pH. Evidence has been obtained for a small conformational change in the molecule, in which titration of the basic NH_2-terminal region produces an "open" form. This can return to the native form at neutral pH, but is maintained at low pH, by neutralization of carboxylate groups in the pepsin portion.

A turbidimetric milk clotting assay for pepsin has been developed. This method is more reproducible, easier and more accurate than other methods and is being used in studies on the mechanism of activation of pepsinogen.

L. Phospholipid Effect on Fructose Phosphate Interconversion in Control of Brain Glycolysis. A regulatory mechanism governing glycolysis in brain has been found as a result of the observation that glucose 6-phosphate (G6-P) accumulates in an unsupplemented supernatant of rat brain and the accumulation can be enhanced by the addition of phospholipid. A study of the fate of the hexose phosphates in the presence and absence of phospholipid shows that phospholipid affects the irreversible interconversion of the fructose phosphates, causing G6-P to accumulate in the wake of inhibition of glycolysis by either stimulation of Fructose 1,6-bis-phosphatase or inhibition of phosphofructokinase or both. The source of G6-P is UDPglucose, itself derived from an endogenous precursor.

M. Particulate Enzymes of Carbohydrate Metabolism. Glucose-6-phosphatase is an enzyme which appears at or immediately before birth and undergoes a rapid proliferation during the first few days of life to quantitative levels many-fold higher than that found in adult animals. This postnatal "overshoot" occurs almost exclusively in the rough membrane fraction, the enzyme in the smooth membranes increasing more gradually with increasing age. A significant portion of the enzyme activity is latent, that is, it is measurable only after "activation" of the membrane by treatment with suitable concentrations of a detergent or controlled alkaline conditions. The earlier observation in adult animals of a much greater degreee of latency for the PP_i-glucose phosphotransferase function of the enzyme than for the hydrolysis of glucose-6-phosphatase had led to propose that the enzyme is so oriented in the membrane that the donor site for glucose-6-phosphate or PP_i is located on the cytoplasmic side while

that for the glucose acceptor is on the luminal side of the endoplasmic reticulum. Results with neonatal animals indicate a high degree of latency for the phosphotransferase soon after birth, quantitatively altogether comparable with the latency seen in the adult animal. In contract, the latency of the hydrolase activity was appreciably great in the neonatal liver than in the adult. The percentage of glucose-6-phosphatase activity tht was latent reached a peak at about three to five days of age and corresponded in time with the maximum quantity of postnatal "overshoot" in absolute enzyme activity in rough membranes.

The study is being extended to include the determination of a number of other enzymes that are known to be characteristic of microsomal membranes. Preliminary results show that both nucleoside diphosphatase, known to occur on the luminal side of the membrane, and NADPH-cytochrome-c reductase, located on the cytoplasmic side, exhibit the same pattern of development with age for the smooth as for the rough membrane fractions. This development differs greatly from that of glucose-6-phosphatase. The results are compatible with the assumption that glucose 6-phosphatase is synthesized <u>in situ</u> on bound ribosomes and then transferred laterally or by ribosome removal to smooth membranes, while the other two enzymes may be made on free ribosomes and subsequently transferred to both rough and smooth membranes.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 17001-10 LBM

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

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

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

PI:	Gilbert Ashwell, Chief, Lab. of Biochem. & Metab.	LBM NIAMDD
OTHER:	Jean Hickman, Research Chemist, Ph.D.	LBM NIAMDD
	Toshisuke Kawasaki, Visiting Fellow, Ph.D.	LBM NIAMDD
	Mr. William Pricer, Research Chemist	LBM NIAMDD

COOPERATING UNITS (if any)

None

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INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

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

It is proposed: 1. To isolate the recently described avian hepatic binding protein in pure form and to determine its physical and chemical properties.

2. To investigate the role of the rat binding protein in intracellular processes with particular emphasis on the mechanism of pinocytosis.

3. To extend the studies on binding and transport of agalacto - and asialoglycoproteins in chicken and rat hepatocytes.

Keyword Descriptors:

Asialoglycoprotein, Galactose, Hepatic binding protein, Hepatic plasma membrane, Neuraminidase, Serum glycoprotein, and Sialic acid.

PHS-6040
(12-75)

Project Description:

Objectives - The major objective of this project has been to investigate the nature of the mechanism whereby hepatic plasma membranes selectively find circulating asialoglycoproteins as a prelude to macromolecular transport and lysosomal catabolism.

Methods Employed -

Hepatic plasma membranes, as well as other intracellular organelles, and intact, viable hepatocytes are isolated by conventional methods of cell fractionation and their binding properties studied with ^{125}I-asialo-orosomucoid as the principal ligand. Solubilization and purification of the binding protein is accomplished by detergent extraction and affinity chromotography.

Major Findings -

The results of continuing studies on the chemical and biological properties of the hepatic membrane protein responsible for the binding of desialylated plasma proteins may be summarized as follows:

1) The purified binding protein isolated from rabbit liver has been shown to be a glycoprotein whose binding activity is destroyed by exposure to neuraminidase. Further information on the nature of the inactivation process was sought by examination of the intrinsic carbohydrate moiety. Two widely different glycopeptides were isolated and their sequence determined. The larger, acidic glycopeptide contained 24 monosaccharide units attached to Asparagine; the smaller, neutral glycopeptide contained 12 monosaccharide units. The disposition of the two classes of glycopeptides on the two non-identical subunits of the hepatic binding protein has been determined. (T. Kawasaki)

2) A methodology has been developed for the detection and quantitation of desialylated glycoproteins in serum and evidence has been obtained to document increased levels of circulating asialoglycoproteins in pstients with impared hepatic function (cirrhosis and hepatitus). These studies have revealed abnormally high levels in avian and reptilian species concomitant with the absence of the specific galactose recognizing hepatic receptor. The evolutionary significance of this finding, has been furthered by the detection of a new avian binding protein which recognizes and binds terminal N-acetylglucosamine residues. (J. Lunney)

Proposed Course of Project -

Studies with intact chicken and rat hepatocytes will be continued in an attempt to clarify the mechanism whereby the binding protein participates in the recognition as well as the uptake and intracellular transport of the ingested material.

Publications:

Kawasaki, T. and Ashwell, G.: Chemical and physical properties of an hepatic membrane protein that specifically binds asialoglycoproteins. J. Biol. Chem. 251: 1296-1302, 1976.

Lunney, J. and Ashwell, G.: A hepatic receptor of avian origin capable of binding specifically modified glycoproteins. Proc. Nat. Acad. Sci. U.S.A. 73: 341-343, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 17002-06 LBM

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Glutathione transferases as catalysts and binding proteins

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

PI: William B. Jakoby, Research Chemist, LBM, NIAMDD
OTHER: Jeanne N. Ketley, Staff Fellow, LBM, NIAMDD
James M. Keen, Staff Fellow, LBM, NIAMDD

COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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

The glutathione S-transferases appear to have three functions: They serve as catalysts in reactions in which glutathione is used to attack a vast number of compounds with a susceptible electrophilic atom; they serve as binding proteins for an even larger number of compounds that include bilirubin and heme; they serve as one of the reactants in the formation covalent complexes with very highly reactive reagents including those with carcinogenic activity. All three functions are under active investigation.

PHS-6040
(12-75)

Project No. Z01 AM 17002-06 LBM

Project Description:

Objectives:

The glutathione S-transferases are among the major detoxication systems of the animal kingdom and perform this function by related mechanisms which all involve binding of protein and ligand. Our work with these enzymes has provided homogeneous transferases from both human and rat liver. The availability of clean systems has allowed extensive studies on the chemical and kinetic mechanisms of the enzymatic reaction as well as the specificity of binding.

Methods Employed:

Studies with these enzymes suggest that they are almost as avid as is albumin in both variety and tenacity with which they will bind ligands. Not only are naturally occurring compounds such as bilirubin and heme bound with dissociation constants of the order of 10^{-6}M, but a vast number of other lipophilic compounds serve as ligands. Current ideas of bilirubin excretion include the proposal that bilirubin is stored, while attached to the transferases intrahepatically but, upon glucuronidation, the conjugate is released at the cell surface and immediately transported through the bile canicull. Our own results (with Allen Wolcoff) negate the hypothesis by demonstrating the presence of large quantities of conjugated bilirubin intrahepatically and by showing that the binding constants of the transferases for bilirubin conjugates are almost as high as those for bilirubin itself.

The catalytic reaction is also being studied with the conclusion that the transferases appear to be primitive enzymes. Because of their avidity in binding, they serve to passively hold compounds with highly electrophilic atoms in close proximity to glutathione. Their active function is in allowing glutathione to ionize at a lower than normal pH and thereby to become the highly reactive nucleophile, glutathione thiolate.

Proposed Course of Project:

Work on glutathione conjugation by enzymes from liver is intimately related to a general mechanism of aromatic oxidation as well as to the biological problem of detoxication of noxious compounds. It is expected that investigations dealing with the above-noted projects will be continued and a study of other model reactions will be initiated.

Publications:

Myers, J. S. and Jakoby, W. B.: Glycerol as an agent eliciting small conformational changes in alcohol dehydrogenase. J. Biol. Chem. 250: 3785-3789, 1975.

Habig, W. H., Keen, J. M. and Jakoby, W. B.: Glutathione S-transferase in the formation of cyanide from organic thiocyanates and as an organic nitrate reductase. Biochem. Biophys. Res. Commun. 64: 501-506, 1975.

Nemoto, N., Gelboin, H. V., Habig, W. H., Ketley, J. N. and Jakoby, W. B.: K-Region benzo(a)pyrene 4,5-oxide is conjugated by homogeneous glutathione S-transferases. Nature 255: 512, 1975.

Cagen, L. M., Pisano, J. J., Ketley, J. N., Habig, W. H., and Jakoby, W. B.: The conjugation of prostaglandin A and glutathione catalyzed by homogeneous glutathione S-transferases from human and rat liver. Biochem. Biophys. Acta 398: 205-208, 1975.

Ketley, J. N., Habig, W. H., and Jakoby, W. B.: Binding of non-substrate ligands to the glutathione S-transferases. J. Biol. Chem. 250: 8670-8673, 1975.

Kamisaka, K., Habig, W. H., Ketley, J. N., Arias, I. M., and Jakoby, W. B.: Multiple forms of human glutathione S-transferase and their affinity for bilirubin. European J. Biochem. 60: 153-161, 1975.

Arias, I. M., and Jakoby, W. B., eds. Glutathione: Metabolism and Function, Raven Press, New York, 1976, 383 pp.

Jakoby, W. B., Habig, W. H., Keen, J. M., Ketley, J. N., and Pabst, M. J.: Glutathione S-transferases: Catalytic aspects. In Glutathione: Metabolism and Function, Raven Press, New York, 1976, pp. 189-211.

Habig, W. H., Kamisaka, K., Ketley, J. N., Pabst, M. J., Arias, I. M., and Jakoby, W. B.: The human hepatic glutathione S-transferases. In Glutathione: Metabolism and Function, Raven Press, New York, 1976, pp. 225-232.

Jakoby W. B., Ketley, J. N., and Habig, W. H.: Glutathione S-transferases: Binding and physical properties. In Glutathione: Metabolism and Function, Raven Press, New York, 1976, pp. 213-223.

Jakoby, W. B.: Glutathione S-transferases: A family of binding proteins which includes ligandin. In Berk, P., Berlin, N., and Watson, C., (Eds.): Chemistry and Metabolism of Bile Pigments. U.S. Govt. Print. Off., in press.

Jakoby, W. B.: The glutathione S-transferases: A triple-threat in detoxication. In In Vitro Metabolic Activation, Elsevier, in press.

Habig, W. H., Pabst, M. J., and Jakoby, W. B.: Glutathione S-transferase AA from rat liver. Arch. Biochem. Biophys., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 17003-09 LBM

PERIOD COVERED

July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Polysaccharides in Morphogenesis

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

PI:	Enrico Cabib, Research Chemist	LBM NIAMDD
OTHER:	Angel Duran, Guestworker	LBM NIAMDD
	Martin Slater, Staff Fellow	LBM NIAMDD
	Eleanor Shematek, Staff Fellow	LBM NIAMDD
	Jesus Molano, Visiting Scientist	LBM NIAMDD
	Rodney Ulane, Staff Fellow	LBM NIAMDD
	James Braatz, Staff Fellow	LBM NIAMDD

COOPERATING UNITS (if any)

Blair Bowers, LCB, NHLI

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NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYLARS:	PROFESSIONAL:	OTHER:
5	5	0

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

The structure and composition of the yeast plasma membrane will be further studied. The purification of solubilized chitin synthetase will be pursued, with a view to determine the minimal size of an enzymatic complex that can manufacture a fibril in vitro. Further efforts will be made towards obtaining mutants defective in chitin synthesis and towards development of a method to detect specifically chitin at the electron microscope level of resolution.

An enzymatic system from yeast that catalyzes the synthesis of a β-glucan will be further studied, in an attempt to understand its requirements and the mechanism of the reaction.

PHS-6040
(12-75)

Project Description:

Objectives:

The projects carried out during the present period deal with the formation of the yeast primary septum and with the biosynthesis of a yeast glucan. hey will be described separately below.

Methods Employed:

The formation of the yeast primary septum. Work on this topic has continued in three different areas, i.e. localization and solubilization of the chitin synthetase zymogen, purification of the chitin synthetase activating factor, localization of chitin in the cell wall and bud scars and search for mutants in the chitin synthetase system.

a) Localization and solubilization of chitin synthetase zymogen. As mentioned in the previous report, the successful preparation of intact plasma membranes permitted to establish that the chitin synthetase zymogen is attached to the membranes. It appears that the enzyme is facing the interior of the cell, because it can be irreversibly inactivated by glutaraldehyde only after yeast protoplasts are lysed and the inner side of the membrane is exposed.

An ultrastructural study of the membranes by freeze-fracture electron microscopy has begun. Preliminary results show that the isolated membranes are similar in structure and particle distribution to those seen in intact protoplasts and intact cells.

After several attempts with a number of detergents, the chitin synthetase zymogen was finally "solubilized" from the membranes with very good yield by the use of 1% digitonin. "Solubilization" is defined here as lack of sedimentability at 200,000 xg for 1 hour. It was gratifying that the solubilized enzyme was still in the zymogen state, i.e. it only showed activity after treatment with trypsin or yeast protease. This result strongly supports the idea that activation of the enzyme is due to a precursor-active form relationship, rather than to an artifactual phenomenon, such as would be the opening by proteolytic action of a vesicle containing chitin synthetase in its inner face. The solubilized enzyme produces insoluble chitin without any added primer. Work is now in progress on the purification of the enzyme, which still appears to consist of a large aggregate.

b) Purification of the chitin synthetase activating factor. As described in the previous report, the yeast protease that converts the chitin synthetase zymogen into its active form has been purified by affinity chromatography on a Sepharose column to which the proteinaceous inhibitor of the enzyme had been attached. The purified preparation had a very unusual behavior

in that it yielded no stainable band in conventional disc gel electrophoresis; however, when the electrophoresis was carried out in 4M urea at acidic pH a single band could be observed. At the same pH, but in the absence of urea a much fainter band was observed, which coincided with proteolytic activity. It was concluded that the purified enzyme is homogeneous under these conditions. The ratio between proteolytic activity and ability to activate the chitin synthetase zymogen was constant throughout purification. A molecular weight of 55,000 was determined for the enzyme by gel filtration and sedimentation in a sucrose gradient. By titration of the activity it was established that the enzyme combines mole to mole with either its proteinaceous inhibitor from yeast or with phenylmethylsulfonyl fluoride. The latter result indicates that the protease contains one active serine group per molecule. The enzyme is also inhibited by very small concentrations of the actinomycete metabolites chymostatin and antipain. A comparison of its specificity with that of trypsin, using a series of synthetic substrates, showed a rather similar general pattern, with some clear differences.

c) Localization of chitin in the cell wall and bud scars. Previous work has supported the notion that chitin forms the primary septum of yeast and that it remains embedded in the bud scars after cell separation. Nevertheless, we still lack a specific method to study the topology of chitin at the level of resolution of the electron microscope, in order to follow the formation of the septum in detail. Since wheat germ agglutinin has a very strong and specific affinity for chitin, it was thought that this lectin would make a good marker for the polysaccharide, after being coupled to ferritin, in order to make it visible under the electron microscope. Therefore, the agglutinin was purified to homogeneity from wheat germ extracts and attached to ferritin by the use of glutaraldehyde. Preliminary results indicate that the ferritin-bound lectin binds to chitin and can be visualized under the electron microscope. An unexpected and troublesome finding was that the agglutinin also binds to yeast glucan, thus interfering with the observation. It appears, however, that this unspecific binding can be prevented by the use of chitin oligosaccharides at concentrations too low to interfere with the much stronger binding to chitin.

d) Search for mutants in the chitin synthetase system. Previous studies with the chitin synthetase inhibitor polyoxin D had indicated that a temperature-sensitive mutant defective in the chitin synthetase system probably would yield lysed cell pairs at the non-permissive temperature. With this idea in mind and using the recently developed method for the screening of lysing mutant, a search for such mutant was continued. Although several strains with the above-mentioned phenotype were found, so far none of them seems to be affected in chitin synthesis. The search continues along the same lines.

Major Findings:

Biosynthesis of a yeast glucan. During investigations on yeast glycogen

synthetase and its regulation, it was found that some radioactivity from labeled UDP-glucose was incorporated in a trichloroacetic-insoluble fraction. This material was not glycogen, because it was resistant to α and β-amylases. On the basis of its resistance to periodate, of the series of oligosaccharides produced by partial acid hydrolysis and of its degradation by a purified β-glucanase, it was identified in a preliminary way as a β-1,3-linked glucan. Since such glucans are major components of the yeast cell wall and appear to impart shape to the yeast cell, it was considered of importance to continue its study. Recently, particulate preparations have been obtained from protoplasts which can convert about one-third of the substrate into product in 20 min, with the use of about 0.2 mg of protein at a 5 mM concentration of UDP-glucose. The activity requires the presence of glycerol and of unknown factor(s) present in boiled yeast extracts. These factors can be substituted in part by the addition of ATP and magnesium ions. Because of these unusual requirements and of the importance of glucan for the structure of the yeast cell wall, the study of this enzymatic system will be actively pursued.

Publications:

Cabib, E. Molecular aspects of yeast morphogenesis. Ann. Rev. Microbiol. 29: 191-214, 1975.

Cabib, E. and Bowers, B. Timing and function of chitin synthesis in yeast. J. Bacteriol. 124: 1586-1593, 1975.

Cabib, E. and Duran, A. Simple and sensitive procedure for screening yeast mutants that lyse at nonpermissive temperatures. J. Bacteriol. 124: 1604-1606, 1975.

Duran, A., Bowers, B., and Cabib, E. Chitin synthetase zymogen is attached to the yeast plasma membrane. Proc. Nat. Acad. Sci., USA 72: 3952-3955, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 17004-08 LBM

PERIOD COVERED

July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Thermodynamic and Kinetic Studies of Protein Structure at Enzyme Mechanisms

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

PI: Dr. Peter McPhie, Research Chemist, LBM, NIAMDD

COOPERATING UNITS (if any)

None

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NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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

Studies on the relationship between protein conformation and enzymic activity are to be continued. Most specifically, we are interested in the changes in conformation and intramolecular interactions accompanying the transfromation of pepsinogen to pepsin. These changes produce an enormous increase in enzymic activity and in the relative stability of the molecules.

PHS-6040
(12-75)

Objectives:

The digestive enzymes, pepsin is synthesized as an inactive zymogen, pepsinogen. It is hoped that studies on the conformation of these two proteins will reveal the mechanism by which proteolytic activity is repressed in the zymogen.

Methods Employed:

Visible and Ultraviolet spectroscopy; rapid kinetic experiments in Aminco T-jump and stop flow apparatus. These have recently been equipped with a Dasar system which greatly facilitates data collection and processing through an interface with the TSO computing system.

Major Findings:

Above pH 8.5, pepsinogen is converted into a form which cannot be activated to pepsin at low pH. Evidence has been obtained for a small conformational change in the molecule, in which titration of the basic NH_2-terminal region produces an "open" form. This can return to the native form at neutral pH, but is maintained at low pH, by neutralization of carboxylate groups in the pepsin portion.

A turbidimetric milk clotting assay for pepsin has been developed. This method is more reproducible, easier and more accurate than other methods and is being used in studies on the mechanism of activation of pepsinogen.

Proposed Course of Study:

By the use of kinetic and chemical modification studies it is hoped to learn more on the nature of conformational changes in pepsinogen and the relationship of these to the development of enzymic activity at low pH. Experiments are also planned on the relative stabilities of pepsinogen and pepsin under a wide variety of conditions.

Publications:

Klee, C. B., Kirk, K. L., Cohen, L. A. and McPhie, P.: Histidine-ammonia lyase, the use of 4-fluorohistidine in identification of the rate determining step. J. Biol. Chem. 250: 5033-5040.

McPhie, P.: The origin of the alkaline inactivation of pepsinogen. Biochemistry 14: 5253-5256, 1975.

McPhie, P.: A turbidimetric milk clotting assay for pepsin. Anal. Biochem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 17005-11 LBM

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

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

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

ER:

Marie N. Lipsett, Research Chemist, LBM, NIAMDD
Joseph D. Cherayil, Visiting Associate, LBM, NIAMDD

COOPERATING UNITS (if any)

Sankar L. Adhya, NCI

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	2.0	0.1

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

Since the position of the genes specifying the two enzymes involved in the formation of 4-thiouridine in E. coli tRNA is roughly known, a search is being made for E. coli mutants lacking one or the other of the enzymes.

The first enzyme in the system is obtainable in homogeneous form, and will be used to investigate the structural requirements in the acceptor tRNA and the nature of the intermediate produced by the enzyme.

PHS-6040
(12-75)

Objectives:

1. To explore the two-enzyme transsulfurase system which forms 4-thiouridine in tRNA, and to determine the role of this nucleotide in the tRNA molecule.
2. To investigate the tRNA of plant pathogenic organisms, particularly with regard to their content of cytokinin-active nucleotides and of thionucleotides. (J. D. Cherayil)
3. To study the thionucleotide pattern in E. coli tRNA after induction of lysogenic phage λ. (J. D. Cherayil and S. L. Adhya)

Methods Employed:

Standard methods were used for separations involving proteins or nucleic acids, i.e., salt fractionation, phase partition, column chromatography, electrophoresis, partition, electrophoresis and column and thin layer chromatography.

Major Findings:

1. A rough mapping of the location of the two enzymes in the 4-thiouridine sulfurtransferase system was carried out by examining the enzyme levels in a series of 44 F' strains of E. coli K12 carrying known segments of duplicated K12 chromosomal material in the episome. Gene dosage effects were observed in two regions, between pur E and lip for Factor A and between his and mglA for Factor C. Deletion mutants in these broad areas are being screened for lack of 4-thiouridine in their tRNA or for lack of the enzymes. In addition, phage P2-induced K12 deletions obtained from various sources are being "cured" of the P2-episome by acridine orange and also screened.

2. Factor A, the first enzyme in the synthesis of 4-thiouridine will bind to Poly U-agarose, but not to Poly A-agarose or agarose alone. This correlates with the fact that Poly U, but not Poly A, can inhibit the thiolation of tRNA. Under specific salt conditions, the pH optimum for binding is 6.4. The method is useful for purification of the enzyme.

3. Joseph Cherayil has found that the tRNAs of plant pathogens which stimulate plant growth contain methylthioribosyl-zeatin, a cytokinin-active zeatin of the type previously reported only in higher plants. The nucleotide was found in the tRNA of Rhizobium leguminosarum, Agrobacterium tumefaciens and Corynebacterium fascians, all of which stimulate plant growth. On the other hand it was absent in Erwinia Amylovora, an organism which is pathogenic without stimulating growth.

4. Several heretofore undiscovered species of thionucleotides have been found in various tRNAs, but not yet identified. Dr. Cherayil, in collaboration with Dr. Adhya of NCI, has found that the induction of λ phage in lysogenic K12 is accompanied by a ten-fold increase in an unidentified minor

thionucleotide in the tRNA. This effect has been traced to one of the λ genes hitherto without a known function.

Proposed course of project:

1. To continue the search for a strain of E. coli deficient in one or the other enzymes needed for 4-thiouridine formation, so that the function of 4-thiouridine may be studied both in the tRNA and in the whole organism, where it may be involved in some sort of regulation mechanism.

2. To work up a large amount of pure Factor A, including the Poly U-agarose step just developed, for definitive characterization and study of the enzyme-tRNA intermediate.

Dr. Cherayil intends to continue work on the projects he has initiated after his return to Bangalore in June, 1976.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 17006-02 LBM

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Function of Membrane Protein Components in Bacteriocin Tolerance

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

PI: John F. Foulds, Research Chemist LBM NIAMDD
OTHER: Tuu-jyi Chai, Staff Fellow LBM NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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

Escherichia coli strains which carry mutations in the tolF or tolG loci have alterations in the protein composition of their outer membranes. A mutation at either loci results in the loss of a different major outer membrane protein.

These mutants are tolerant to colicin L - JF246 and resistant to at least one bacteriophage.

The goal of the research proposed is to study the synthesis, assembly and biological role of the proteins missing in tolF and tolG mutants. We have purified protein preparations which we have characterized chemically and hope to develop a biological assay using the purified material to reconstitute bacteriocin and/or bacteriophage sensitivity in tolerant mutants.

PHS-6040
(12-75)

Project Description:

Objectives - The goals of this project were to purify the tolG protein, a major component of the outer membrane of E. coli, in sufficient quantities to permit chemical and biological characterization. We hoped to use this material to reconstitute bacteriocin sensitivity in bacteriocin-tolerant mutants. We also wished to complete the genetic, physiological and chemical characterization of tolF strains, a new class of colicin-tolerant mutants.

Methods Employed -

1. We have purified a major outer membrane protein (the tolG protein, missing in tolG mutants) by preparative polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The apparent molecular weight, amino acid composition, N-terminal amino acid, phosphate, carbohydrate and lipid content of the purified material was determined.

2. The biological activity of the purified protein was tested by determination of its ability to inactivate phage K_3 or colicin L-JF-246.

3. Incorporation of membrane proteins into unilamellar phospholipid vesicles was accomplished by extensive sonication of an excess of purified phospholipid in the presence of cell envelope proteins.

4. The role of phospholipid composition in colicin sensitivity was studied using mutants defective in phospholipid biosynthesis.

5. The role of tolG protein in the mode of action of colicin L-JF 246 was studied by defining conditions which alter the colicin sensitivity of cells or membrane vesicles.

6. The cell envelope of tolF mutants was separated by isopycnic centrifugation in a sucrose gradient into fractions that contained primarily inner or outer membranes. The protein composition of these fractions was examined by high resolution gel electrophoresis and compared with that of similar material prepared from wild type tol^+ cells.

Major Findings -

1. The purified tolG protein is apparently identical with protein II*, purified by Henning and coworkers and protein B, purified by Bragg and coworkers. The following findings apply equally to all three preparations. (T. Chai)

Major Findings - (Continued)

The tolG protein has an unusual property in that its mobility on polyacrylamide gels containing SDS depends upon how the protein was heated prior to applying to the gel. The apparent molecular weight is about 28,500 when heated at 50° whereas this value is increased to 33,500 when heated at 100°. The amino acid composition is not remarkable, the N-terminal amino acid is alanine and the purified material contains 2 moles of carbohydrate and no detectable lipid or phosphate. The purified material has no detectable biological activity even when the SDS is removed. Evidently the protein was irreversibly denatured by the SDS treatment.

2. We have found conditions where up to 70% of the cell membrane proteins can be incorporated into unilamellar phospholipid membrane vesicles. Mixing these vesicles with intact cells results in an association of the added membrane proteins with the cells.

This finding, although preliminary, augers well for the incorporation of exogenous (purified) membrane proteins into intact cells. Similar unilamellar phospholipid vesicles have been shown to fuse with the outer membrane of E. coli cells. Our goal here is to define conditions where we can reconstitute colicin and/or bacteriophage sensitivity in tolerant cells by adding the missing membrane protein. Evidently unilamellar phospholipid vesicles are an appropriate vehicle for this transfer.

3. The tolF locus was shown to be phenotypically and genetically identical to the cmlB locus. A manuscript describing these findings has been submitted to the Journal of Bacteriology.

tolF strains, like tolG strains, have been found to be missing a major outer membrane protein. The proteins missing in the two strains are distinct.

4. tolG cells are tolerant to colicin L-JF 246. The most prominent alteration associated the mutation to tolerance is the loss of the tolG protein from the outer membrane. Tolerance in tolG cells can be overcome by a variety of techniques, each of which has the common feature that the integrity of the outer membrane is perturbed. These results suggest that there may be a direct interaction of colicin L-JF 246 with the cytoplasmic membrane and the function of the tolG protein may be to assist the colicin molecule, or a portion of it, through the outer membrane barrier. This material will be presented at the annual meeting of the American Society of Biological Chemist.

Proposed Course of the Project -

There are two major long term goals of this project. First, to study the synthesis of certain protein components of biological membranes and second, to define the role of these proteins both in the normal physiology of the cell and the sensitivity of the cell to colicins and bacteriophages.

Significance to Bio-medical Research and the Program of the Institute-

Our short term objectives are to use purified cell membrane components to reconstitute colicin and/or bacteriophage sensitivity to bacteriocin-tolerant, phage resistant cells. To accomplish this we must purify individual outer membrane proteins in a functional form, isolate phospholipids from E. coli, define conditions for the incorporation of these components into biological membranes, quantitate the extent of this incorporation, and finally, design an assay to reveal the functional reconstitution of colicin or bacteriophage sensitivity.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-18000-11 LBM

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Hormone-Dependent Differentiation of Mammary Gland

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

PI:	Dr. Yale J. Topper	Research Chemist, Chief, Section on Intermediary Metabolism	LBM NIAMDD
Other:	Dr. Takami Oka	Research Chemist	LBM NIAMDD
	Dr. Barbara K. Vonderhaar	Senior Staff Fellow	LBM NIAMDD
	Dr. Colette S. Freeman	Staff Fellow	LBM NIAMDD
	Dr. Lech Zwierzchowski	Visiting Fellow	LBM NIAMDD

COOPERATING UNITS (if any)
Dr. M. Wilchek, CE NIAMDD

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.75	3.75	2.0

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

Specifically guanidinated insulin, prolactin and placental lactogen are more active biologically than the corresponding native hormones on mouse mammary epithelium *in vitro*. The modified insulin is also more active than the native hormone on fat tissue and diaphragm from insulin-resistant obese mice. L-thyroxine and L-triiodothyronine enhance differentiative function induced by insulin, glucocorticoid and prolactin in mammary explants. They do not enhance non-differentiative functions. It appears that this action of the thyroid hormones is associated with that of prolactin.

PHS-6040
(12-75)

Project Description:

Objectives - To study the molecular and cytological phenomena involved in development of the mammary gland. This includes attempts to understand the mechanism of action of insulin, prolactin, placental lactogen, glucocorticoids, estrogen and progesterone.

Methods Employed -

A. In Vitro. Mammary gland explants, mammary epithelial cells or mammary cell nuclei are cultured in synthetic media. Synthesis of α-lactalbumin is followed by measuring the formation of lactose in tissue extracts. DNA and RNA synthesis are studied using conventional methods. DNA synthesis is inhibited by the addition of 1-β-D-Arabinosylcytosine or fluorodeoxyuridine. Insulin-Sepharose, prolactin-Sepharose and placental lactogen-Sepharose are made by conventional methods. The intra-cellular accumulation of α-aminoisobutyric acid (AIB) is followed with the use of ^{14}C-AIB or ^{3}H-AIB. Glucose oxidation is assessed by measuring the conversion of ^{14}C-Glucose to $^{14}CO_2$.

B. In Vivo. Male mice are treated with various hormone combinations. Growth of the mammary epithelium is monitored in stained, whole-mount preparations. Differentiation is monitored by assaying for α-lactalbumin activity.

Major Findings -

A. It was reported last year that super-active forms of insulin, prolactin and placental lactogen can be released from the corresponding Sepharose complexes by treatment with proteins such as bovine serum albumin. It has since been observed that similar super-active hormones are also released when the nucleophile is NH_3. In this instance, monosubstituted guanidines (guanidinated hormones) are formed. The identity of the amino acid residue of the hormones which adds to the activated Sepharose, leading ultimately to the formation of the soluble, super-active hormones, has not been determined. The small fraction of the total bound protein which gives rise, during treatment with NH_3, to hormones with enhanced biological material is not bound via lysine, since the released materials contain no detectable homoarginine.

B. The epithelium in the mammary gland of the adult male Swiss NIH mouse resembles the anlage in the embryonic female. We have used the male animal as a convenient source, then, of embryonic-like mammary epithelium. It has been observed that appropriate hormone treatment in vivo can elicit growth and differentiation of the male epithelium equivalent to that which occurs in the pregnant female. Such growth is not dependent upon insulin, but functional differentiation is dependent upon the hormone.

C. L-thyroxine (T_4) and L-Triiodothyronine (T_3) (but not the D-forms)

evoke a three- to five-fold augmentation of α-lactalbumin activity induced in mammary explants in the presence of insulin, glucocorticoid and prolactin. Maximal effects are produced by 10^{-8} M T_4 and 10^{-10} M T_3. The accumulation of α-aminoisobutyric acid, glucose-6-phosphate dehydrogenase activity, galactosyl transferase activity and DNA synthesis are not affected by the thyroid hormones. The effect on α-lactalbumin appears to reflect an increased sensitivity of the cells to prolactin, but not to insulin or glucocorticoid.

Proposed Course of Project -

A. It will be of interest to determine whether modifications of polypeptide hormones, similar to those described, occur physiologically at peripheral sites.

B. The mammary epithelium of adult male mice will be used as a model system for studying growth-control of embryonic-like cells.

C. Differentiating mammary cells will be used *in vitro* to study certain aspects of the action of thyroid hormones.

Significance to Bio-medical Research and the Program of the Institute - Hormones are involved in normal growth and development and are implicated in many disease states. The mammary gland system offers an excellent opportunity to increase our understanding about the ways in which a number of different types of hormones interact with target cells.

Publications:

Oka, T. and Topper, Y. J.: Insulin-unresponsive tissues respond to super-active insulin-like material. Science 188: 1317-1319, 1975.

Topper, Y. J., Oka, T., Vonderhaar, B. K. and Wilchek, M.: Characterization of super-active insulin, prolactin and placental lactogen. Biochem. Biophys. Res. Commun. 66: 793-798, 1975.

Vonderhaar, B. K.: A role of thyroid hormones in differentiation of mouse mammary gland *in vitro*. Biochem. Biophys. Res. Commun. 67: 1219-1225, 1975.

Topper, Y. J., Oka, T., Vonderhaar, B. K. and Wilchek M.: An insulin-derivative with biological activity greater than that of native insulin. J. Cellular Physiology, in press.

Topper, Y. J., Oka, T. and Vonderhaar, B. K.: Techniques for studying development of normal mammary epithelial cells in organ culture. In Hardman, J. G. and O'Malley, B. W. (Eds.): Methods in Enzymology, New York, Academic Press, Vol. XXXIX, 1975, pp. 443-454.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18001-02 LBM

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Phospholipid Effect on Fructose Phosphate Interconversion in Control of Brain Glycolysis

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

PI: Dr. Frank Eisenberg, Jr. Research Chemist LBM NIAMDD

Other: Dr. Arunendra L. Majumder Visiting Fellow LBM NIAMDD

COOPERATING UNITS (If any)

Dr. M. Hokin Neaverson, Dr. K. Sadeghian: Department of Psychiatry and Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 54306

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Intermediary Metabolism

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

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

A regulatory mechanism governing glycolysis in brain has been found as a result of the observation that glucose 6-phosphate (G6-P) accumulates in an unsupplemented supernatant of rat brain and the accumulation can be enhanced by the addition of phospholipid. A study of the fates of the hexose phosphates in the presence and absence of phospholipid shows that phospholipid affects the irreversible interconversion of the fructose phosphates, causing G6-P to accumulate in the wake of inhibition of glycolysis by either stimulation of fructose 1,6-bis-phosphatase or inhibition of phosphofructokinase or both. The source of G6-P is UDPglucose, itself derived from an endogenous precursor.

PHS-6040
(12-75)

Project Description:

Objective - Elucidation of the source of G6-P and the role of phospholipid in the accumulation of G6-P in brain supernatant.

Methods - A low speed supernatant of brain was incubated alone and with phospholipids or fatty acids and the concentration of G6-P measured by gas-liquid chromatography of the trimethylsilyl ether. Labeled precursors were added and labeled G6-P was isolated by preparative gas-liquid chromatography and counted. The fates of labeled G6-P and other glycolytic intermediates were studied by paper chromatography.

Major Findings - The primary observations are the accumulation of G6-P in unsupplemented brain low speed supernatant, in contrast to its rapid disappearance in intact anoxic brain, and the enhancement of this accumulation at least ten-fold by the addition of phospholipid. The sources of G6-P are glycogen, UDPglucose, and glucose 1-phosphate, all of which are in turn derived from an endogenous precursor. Phospholipid inhibits the degradation of G6-P by the glycolytic cycle at the level of the irreversible interconversion of the fructose phosphates. The evidence for this conclusion is that in the absence of phospholipid labeled G6-P and fructose 6-phosphate (F6-P) largely disappear and fructose 1,6-diphosphate (FDP) accumulates; in the presence of phospholipid the monophosphates accumulate, G6-P predominating in accordance with the equilibrium established by isomerase, and FDP disappears.

Significance to Biomedical Research and the Program of the Institute - Regulation of the flow of metabolites is an important aspect of cellular chemistry and its derangement can lead to pathological states. Since G6-P is the primary fuel of brain its proper partition among the various pathways of utilization is important to the function of that organ. Regulation of glycolysis is required in order to conserve G6-P for other needs, e.g. the biosynthesis of inositol, the major neutral sugar of brain.

Proposed Course of Project - The irreversible interconversion of F6-P and FDP is mediated by fructose bisphosphatase and phosphofructokinase. The effect of phospholipid can be either stimulation of the former, inhibition of the latter, or both. We have preliminary evidence for the presence of the phosphatase in brain, a question that has long been the subject of controversy. The isolation of the enzyme will be attempted and if successful the effect of phospholipid will be studied.

Publications:

Hokin-Neaverson, K., Sadeghian, K., Majumder, A. L. and Eisenberg, F., Jr.: Inositol is the water-soluble product of acetylcholine-stimulated breakdown of phosphatidylinositol in mouse pancreas. Biochem. Biophys. Res. Commun. 67: 1537-1544, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18002-03 LBM

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Investigations on the Biochemical Abnormality Underlying Human Cystinosis

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

PI: Dr. Frank Tietze Research Chemist LBM NIAMDD

COOPERATING UNITS (if any)

Dr. Joseph D. Schulman, Dr. E. Jean Butler: Section on Human Biochemical and Developmental Genetics, NPMB, NICHHD.

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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

Several fibroblast cell lines from patients with the rare lysosomal storage disease mucolipidosis II (I-cell disease) have been found to possess markedly elevated intracellular levels of cystine. In contrast, the intracellular cystine contents of two cell lines from patients with the similar, but clinically less severe, mucolipidosis III were within normal limits. The identity of excess free cystine in I-cells was confirmed by several independent assays including automated amino acid analysis, high-voltage paper electrophoresis, and by a specific cystine-binding protein assay. The finding of elevated cystine in I-cells may contribute to an understanding of the pathogenesis of human cystinosis. Studies on additional cell lines from patients with mucolipidosis II will be needed to determine whether elevated intracellular free cystine is an invariable concomitant of this disease.

PHS-6040
(12-75)

Project Description:

Objectives - To define the biochemical basis of cystinosis, a rare and often fatal inherited disease of children and young adults characterized by the abnormal accumulation of the amino acid cystine within lysosomes of certain cells of the body.

Methods Employed - Skin fibroblasts from cystinotic patients and from individuals with certain unrelated lysosomal storage diseases are grown in tissue culture by standard procedures. Cells are washed, disrupted, and analyzed for their intracellular content of cystine on an amino acid analyzer or by some other appropriate procedure.

Major findings - Several different cell lines from individuals with the lysosomal storage disease mucolipidosis II (I-cell disease) have been found to contain abnormally high intracellular levels of cystine (1-5 nanomoles/mg protein), approaching the levels usually encountered in cystinotic cells (5-15 nanomoles/mg protein). The identification of cystine in these I-cells was established by: (1) automated amino acid analysis; (2) appearance of a prominent radioactive peak corresponding in position to authentic cystine following high voltage electrophoresis of extracts of cells incubated in the presence of ^{35}S-cystine; (3) radioassay with the specific cystine-binding protein derived from E. coli. The intracellular component was further established as cystine, and not cysteine, by disruption of the cells in the presence of N-ethylmaleimide, a specific thiol-binding agent. Crude fractionation of extracts of I-cells with high intracellular cystine by differential centrifugation showed that, as in the case of cystinotic cells, a significant proportion of cystine was localized in the granular fraction. Several fibroblast cell lines from individuals with the related but less severe storage disease known as mucolipidosis III were examined and found to contain normal amounts of cystine.

Significance to Biomedical Research and the Program of the Institute - Cells from individuals with mucolipidosis II or mucolipidosis III are characterized by abnormal intra-lysosomal accumulations of polymeric material of diverse nature as a result of multiple deficiency of acid hydrolases. The finding that several of the type-II lines (I-cells) also contain abnormally high cystine levels suggests that the intracellular disposition of this amino acid is at least partly dependent on some lysosomal factor, the nature of which is unknown. The observations made with I-cells may have implications with respect to the pathogenesis of human cystinosis.

Proposed Course of Project - Studies will continue on the possible relationship of elevated cystine levels in cystinotic and I-cell fibroblasts. In particular, it will be of interest to examine whether the abnormal intracellular levels of cystine in either or both cell types can be "corrected" (i.e., reduced) by exposure of such cells to conditioned medium from normal cells in a manner similar to that observed with the abnormal accumulation

of intermediate metabolites in several other lysosomal storage diseases.

Publications:

Schulman, J. D., Goodman, S. I., Mace, J. W., Patrick, A. D., Tietze, F. and Butler, E. J.: Glutathionuria: Inborn error of metabolism due to tissue deficiency of gamma-glutamyl transpeptidase. Biochem. Biophys. Res. Commun. 65: 68-74, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-18003-03 LBM

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies on the role of spermidine in the development of mammary gland.

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

PI:	Dr. Takami Oka	Research Chemist	LBM NIAMDD
Other:	Dr. Kazutaka Kano	Visiting Fellow	LBM NIAMDD
	Mr. John W. Perry	Research Assistant	LBM NIAMDD

COOPERATING UNITS (If any)

None

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Intermediary Metabolism

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.75	1.75	1.0

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

The interplay of insulin, glucocorticoid and prolactin induces the development of mammary epithelium *in vitro* by converting non-secretory mammary epithelium into secretory cells. Previous studies on this system indicated that the biosynthesis of spermidine, a naturally-occurring polyamine, may be an important regulatory step in lactogenesis. To elucidate the control mechanism for spermidine biosynthesis, we have studied ornithine decarboxylase, which catalyzes the formation of putrescine, a precursor of spermidine, and which is considered to be a rate-limiting enzyme in the biosynthesis of spermidine. The data show that the enzyme activity in mammary cells increases biphasically during the culture with three hormones. The first peak of activity occurs independently of the added hormones, whereas the second peak depends on the action of insulin and prolactin. Additional studies indicate possible involvement of cyclic AMP as a stimulant of the first hormone-independent peak of activity.

Based on our previous observation that addition of spermidine with insulin and prolactin elicits a marked stimulation of milk protein synthesis in cultured mammary cells, we have studied the uptake and metabolism of exogenous spermidine. The data reveal the existence of a transport system for spermidine in mammary epithelium. Some features of the transport system and subsequent metabolism of the polyamines are described.

Project Description:

The polyamine spermidine is present ubiquitously in living cells, and is currently considered to play an important regulatory role in the process of growth and development of mammalian cells.

In mammary epithelium, the concentration of spermidine increases markedly during lactation. A similar increase has been found during the hormonal induction of lactogenesis in vitro, which is effected by cultivation of mouse mammary explants with insulin, glucocorticoid and prolactin. Previous studies on the role of spermidine with this in vitro system have presented several lines of evidence indicating that the polyamine may serve a vital function in milk protein synthesis, possibly by mediating the effect of glucocorticoid.

Based on these previous findings, we have begun a series of studies to elucidate the mechanism of hormonal control of spermidine formation during mammary development in vitro. Since the major biosynthetic pathway of spermidine in cultured mammary cells probably involves the following steps, i.e., arginine $\longrightarrow$ ornithine $\longrightarrow$ putrescine $\longrightarrow$ spermidine, we have examined the effect of hormones on the enzymes which catalyze each of these steps.

The experimental procedures employed in this investigation include organ culture of mouse mammary gland, preparation of mammary epithelium free of fat cells, isolation and quantification of the polyamines, enzyme assay for ornithine decarboxylase, S-adenosyl-L-methionine decarboxylase, arginase, and spermidine synthetase. In addition, a number of column chromatography techniques, such as DEAE, Sephadex, hydroxyapatite, and affinity chromatography have been used. The details of these procedures are described in the list of publications.

Recent studies have shown that prolactin, in the presence of insulin, causes a several-fold increase in the activity of arginase, an enzyme which catalyzes the formation of ornithine. More recently, it has been found that mouse mammary epithelium possesses two forms of arginase, one being particulate-bound and the other in the soluble fraction. The stimulatory effect of prolactin was largely on the soluble form of the enzyme, and this enzyme appears to be involved in the polyamine biosynthesis.

The activity of ornithine decarboxylase, which converts ornithine to putrescine, increases biphasically with one peak being elicited simply by incubating the tissue in a synthetic medium and the second peak being stimulated by both insulin and prolactin. Several lines of evidence suggest the involvement of cyclic AMP as a stimulant of the first peak of activity.

Although the data have not been published, we have also studied the

the hormonal regulation of S-adenosyl-L-methionine decarboxylase and spermidine synthetase, the two enzymes which are involved in the final step of spermidine biosynthesis. The former enzyme has been purified to an apparent homogeneity and its properties have been studied.

In addition, based on the previous finding that exogenously added spermidine effectively mimicks the effect of glucocorticoid on milk-protein synthesis, we have studied the uptake and metabolism of exogenous spermidine in cultured mammary cells. These studies have led us to discover the existence of a transport system for spermidine in mammary epithelium. Some features of this transport system have been described, and its possible importance in the regulation of the cellular level of the polyamine has been discussed.

The results presented above, together with earlier findings, indicate that the concentration of spermidine in mammary epithelium may be regulated by a number of factors including insulin, glucocorticoid, prolactin and possibly cyclic AMP, which interact to stimulate a group of polyamine biosynthetic enzymes, as well as a polyamine transport system. Such hormonal interplay may be fundamental for the regulation of lactogenesis, since spermidine has been implicated as one of the regulatory agents involved in the development of mammary epithelium.

We intend to pursue further the elucidation of the regulatory mechanism for the biosynthesis of spermidine, and to attempt to dissect the mechanisms of action of spermidine at subcellular and molecular levels. In view of growing evidence for the critical role of this polyamine in tissue growth and development, as well as in various pathophysiological states, including cancer, an understanding of its function may serve to reveal the complex regulatory mechanism of these processes.

Publications:

Oka, T.: Hormonal control of the development of mammary gland. (In Japanese.) Kagaku (Science - Japan) 45: 400-409, 1975.

Oka, T. and Perry, J. W.: Studies on regulatory factors of ornithine decarboxylase activity during development of mouse mammary epithelium in vitro. J. Biol. Chem. 251: 1738-1744, 1976.

Kano, K. and Oka, T.: Polyamine transport and metabolism in mouse mammary gland. General properties and hormonal regulation. J. Biol. Chem., 251: 2795-2800, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18004-03 LBM

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biochemical Lesions in the Genetic Mucopolysaccharidoses

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

PI: Dr. Irwin G. Leder Research Chemist LBM NIAMDD

COOPERATING UNITS (if any)

Section on Human Biochemical Genetics, ARB, NIAMDD

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Intermediary Metabolism

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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

Two mucopolysaccharidoses, the Hunter syndrome and the Hurler syndrome, are lysosomal disorders due to the absence, respectively, of iduronate sulfatase and iduronidase. Specific substrates have been prepared and radiological assay procedures for the purification and study of these enzymes and for the diagnosis of the corresponding genetic diseases have been developed. A corrective form of iduronidase, efficiently internalized by fibroblasts in tissue culture, has been separated from a non-corrective form by affinity chromatography.

PHS-6040
(12-75)

Project Description:

Objectives - To study the hereditary biochemical defects of lysosome dysfunction which are responsible for a group of interrelated mucopolysaccharidoses and to apply the results of such studies to clinical diagnosis and therapy.

Methods Employed - Iduronidase and iduronate sulfatase are purified from human urine and, in the case of iduronidase, from human kidney, by standard techniques and by affinity chromatography. Substrates are prepared by chemical depolymerization of heparin and radioactive labeling of the appropriate disaccharide units. The characteristics of the purified enzymes and their specific uptake by cultured fibroblasts are studied.

Major Findings -

1. Previous studies have indicated the existence of two forms of iduronidase, corrective and non-corrective for the Hurler syndrome in cell culture. By means of affinity chromatography, it has been possible to separate the two forms; only the corrective form, of higher molecular weight, is efficiently internalized by Hurler fibroblasts.

2. A new assay for iduronidase, based on the hydrolysis of tritium-labeled iduronosyl-anhydromannitol, has been applied to the purification of the enzyme and to the diagnosis of the Hurler defect.

Significance to Biomedical Research and the Program of the Institute - The development of techniques for studying the genetic mucopolysaccharidoses is of medical significance in terms of diagnosis, genetic counseling and possible treatment. The availability of specific and sensitive biochemical assays for iduronidase and iduronate sulfatase has simplified the task of diagnosing the Hunter and Hurler syndromes and distinguishing them from other mucopolysaccharide storage diseases. The purification of these enzymes from human sources may lead to clinical trials in enzyme replacement therapy.

Proposed Course of Project -

1. The Sanfilippo A disease is caused by a deficiency of a heparan-N sulfatase. It has been difficult to study because of the insensitivity and other problems inherent in the only available assay procedure which utilizes heparin as substrate. Simple N-sulfated glucosaminides will be prepared from glucosamine and from chitosan and tested as substrates for this enzyme. Assay and diagnostic procedures based upon the release of $^{35}SO_4$ and the appearance of amino groups will be developed.

2. The iduronidase substrate, iduronosyl anhydromannitol, will be made available to other laboratories for diagnostic purposes.

Publications:

Leder, I. G.: Thiamine, biosynthesis and function. In D. M. Greenberg, (Ed.) Metabolism of Sulfur Compounds, New York, Academic Press, Vol. VII, 3rd ed., 1975, pp. 57-85.

Shapiro, L. J., Hall, C. W., Leder, I. G. and Neufeld, E. F.: The relationship of α-L-iduronidase and Hurler corrective factor. Arch. Biochem. and Biophys. 172: 156-161, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18005-03 LBM

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Differentiation of Lymphoid Cells

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

PI:	Dr. Milton Kern	Research Chemist	LBM NIAMDD
Other:	Dr. Nobukata Shinohara	Visiting Fellow	LBM NIAMDD

COOPERATING UNITS (if any)

Dr. Daniel H. Zimmerman, Electronucleonics Laboratories, Bethesda, Md. 20014

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

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Section on Intermediary Metabolism

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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

Rabbit spleen cells, cultured *in vitro*, undergo differentiation into IgM-producing cells in the absence of antigenic stimulation. Addition of lipopolysaccharide (LPS) results in a several-fold enhancement of DNA synthesis as well as a several-fold enhancement in induction of immunoglobulin production. Previous reports by others concerned with the mechanism of action of LPS favored either B or T cells as the target site for LPS. The present results account for this controversy by demonstrating that B-cells are the direct target for the mitogenic activity of LPS; presumably resulting in cell proliferation, and T-cells are required to stimulate some B-cell differentiation event.

A selective anti-mitogenic component of normal serum which also suppresses the induction of immunoglobulin production has been purified and partially characterized. The inhibitor is specific for B cells rather than T cells or adherent cells, e.g. macrophages, and the data indicate that the inhibition of DNA synthesis and suppression of immunoglobulin production are related. Moreover, the serum inhibitor (anti-mitogen) and LPS (mitogen) somehow compete as regards their opposite effects on the induction of immunoglobulin production. It is suggested that the serum inhibitor functions as a homeostatic control *in vivo*.

PHS-6040
(12-75)

Project Description:

Objectives - The purpose of this project is to study the differentiation of the cells involved in the immune response with regard to cell types, cell interactions and control mechanisms. Emphasis during this report period was given to an analysis of an inhibitor present in normal serum which suppresses the induction of immunoglobulin-producing cells as well as an analysis of the mechanism by which lipopolysaccharide (LPS) stimulates the induction of immunoglobulin-producing cells.

Methods Employed - Rabbit lymphoid cells were incubated in tissue culture medium for 72 hours at 37° C. The cells were then harvested, washed in leucine-free medium and incubated in such medium supplemented with [^{3}H]-leucine for 90 minutes. Immunoglobulin production was judged from the quantity of [^{3}H]-immunoglobulins secreted into the extracellular fluid. [^{3}H]-Immunoglobulins were assessed by coprecipitation in the presence of carrier rabbit immunoglobulin and antisera specific for rabbit immunoglobulin. DNA synthesis was analyzed by following the incorporation of [^{3}H]-thymidine into DNA.

Major Findings - Rabbit lymph or spleen cell populations cultured *in vitro* in the presence of fetal calf serum are induced to produce immunoglobulin M-secreting cells. The induction of such immunoglobulin production as well as DNA synthesis was inhibited when cells were cultured with sera from a variety of species despite the presence of fetal calf serum. An early event in the induction process is affected by serum because its addition at 36 hours was ineffectual and the presence of serum for only the first 24 hours yielded the same inhibition as the presence of serum throughout the tissue culture period.

The induction of immunoglobulin production and DNA synthesis were equally inhibited by the same range of serum concentrations. Unlike conventional inhibitors of DNA synthesis, the inhibitory sera exhibited selective specificity with regard to the kinds of cells that could be affected. From this and other data it has been demonstrated that bone marrow (B) cells rather than thymus-derived (T) cells or adherent cells, are the site of action of the inhibitor.

The sera of all species examined were inhibitory except for fetal sera. Ascites fluid and lymph node extracellular fluids contained less inhibitor than found in the serum of the same animal and lysates of washed lymph node cells were devoid of inhibitor. Although fetal bovine serum and newborn bovine serum did not contain the inhibitor, it was detectable within 24 hours of parturition.

B-cells have been demonstrated to be the direct target of LPS-enhanced induction of immunoglobulin production, while T-cells have a regulatory role. Thus, following depletion of the T-cells of spleen with anti-thymocyte serum, the enhancement of immunoglobulin production of the treated cells by LPS was found to be dependent on the number of thymocytes added. Moreover, the prior

incubation of T-cell-depleted spleen cells with LPS resulted in effective enhancement of immunoglobulin production when such T-cell-depleted spleen cells and thymocytes were combined after removal of LPS. On the other hand, an identical experiment, except that thymocytes instead of T-cell-depleted spleen cells received the prior incubation with LPS, did not result in enhancement of immunoglobulin production.

A relationship between the effect of LPS on immunoglobulin production and the effect of LPS on DNA synthesis was indicated by the findings as follows: (1) the optimal dose of LPS was the same for both activities, (2) the enhancement of immunoglobulin production by LPS was invariably accompanied by increased DNA synthesis, and (3) when DNA synthesis was inhibited, enhanced immunoglobulin production was inhibited. It should be noted that the apparent requirement for DNA synthesis is consistent with a proliferative function for LPS on B-cells.

Significance to Biomedical Research and the Program of the Institute - A knowledge of the control mechanisms involved in the induction of immunoglobulin production will have vital importance not only in understanding how the organism reacts to foreign antigens, but also may be expected to significantly affect the approach used in regard to a number of immune diseases, e.g., immunodeficiency disease, allergic disease, autoimmune disease and neoplastic disease of lymphoid cells.

Proposed Course of Project - Efforts will be directed towards studying the binding of the active component in LPS (lipid A) in order to study the site on B-cells that triggers the mitogenic response and the mechanism by which the membrane perturbation results in DNA synthesis. The effect of the normal serum suppressor on the primary and secondary immune response will be studied in order to evaluate preliminary findings which suggest that the primary response is inhibited but that the secondary response is not.

Publications:

Kern, M.: Surface immunoglobulins: Characteristics, mobility and role in immune phenomena. In Jamieson, G. A. and Greenwalt, P. J. (Eds.): Mammalian Cell Membranes. London, Butterworth & Co., in press.

Okumura, K., and Kern, M.: Differentiation of lymphoid cells: suppression of the induction of immunoglobulin M secreting rabbit lymph node cells by cells present in bone marrow. Cellular Immunology 17: 19-29, 1975.

Okumura, K., and Kern, M.: The absolute requirement for T-cells in the induction of IgM secreting cells, in vitro. Ann. N.Y. Acad. Sci. 249: 477-483, 1975.

Zimmerman, D. H., and Kern, M.: Differentiation of lymphoid cells:

A selective anti-mitogenic component of normal serum which inhibits the induction of immunoglobulin production. J. Biol. Chem. 251: 2469-2474, 1976.

Shinohara, N. and Kern, M.: Differentiation of lymphoid cells: B cell as a direct target and T cell as a regulator in lipopolysaccharide enhanced induction of immunoglobulin production. J. Immunol., in press.

Shinohara, N. and Kern, M.: Differentiation of lymphoid cells: The non-mitogenic induction of immunoglobulin production by thymus cell extract and thymus cell culture filtrate. Immunology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18006-05 LBM

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Particulate Enzymes of Carbohydrate Metabolism

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

PI:	Dr. Marjorie R. Stetten	Research Chemist	LBM NIAMDD
Other:	Mr. Paul K. Goldsmith	Research Assistant	LBM NIAMDD

COOPERATING UNITS (If any)

None

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

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Section on Intermediary Metabolism

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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

Studies of 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 have been continued. An enzyme has been found in Limulus polyphemus which resembles vertebrate glucose-6-phosphatase in its specific anatomical distribution, pH optimum, kinetic properties, phosphotransferase activity, substrate specificity and phospholipid dependence. A variety of other invertebrates tested exhibited little or no enzyme with these properties. The hypothesis that a specific glucose-6-phosphatase is to be found only in those animals which utilize free glucose as an important circulating form of energy has been proposed. It appears that a variety of transport compounds, such as trehalose and glucose was tried at the evolutionary level of the Arthropods. The development of glucose-6-phosphatase and related enzyme activities in the liver and kidneys of neonatal rats has been studied as a function of age.

PHS-6040
(12-75)

Project Description:

The objective of the project has been to study the occurrence, properties and functions of some of the membrane-bound enzymes of liver and kidney which catalyze the synthesis, hydrolysis and transport of a variety of phosphorylated sugars and sugar alcohols. 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.

We are continuing our studies of the development of glucose-6-phosphatase and its related enzyme activities in the endoplasmic reticulum of the livers and kidneys of young rats in the hope of contributing to an understanding of the mechanism of synthesis of biological membranes. A series of experiments has been completed in which the quantity of enzyme and its latency properties have been determined, as a function of the age of the animals, on whole homogenates and on microsomal subfractions of liver and kidney homogenates. A CsCl-sucrose density gradient method has been explored and used for the effective separation of smooth and rough membranes from the plasma membrane fractions which usually contaminate such preparations.

Glucose-6-phosphatase is an enzyme which appears at or immediately before birth and undergoes a rapid proliferation during the first few days of life to quantitative levels many-fold higher than that found in adult animals. We have found that this postnatal "overshoot" occurs almost exclusively in the rough membrane fraction, the enzyme in the smooth membranes increasing more gradually with increasing age. A significant portion of the enzyme activity is latent, that is, it is measurable only after "activation" of the membrane by treatment with suitable concentrations of a detergent or controlled alkaline conditions. Our earlier observation in adult animals of a much greater degree of latency for the PP_i-glucose phosphotransferase function of the enzyme than for the hydrolysis of glucose-6-phosphatase had led us to propose that the enzyme is so oriented in the membrane that the donor site for glucose-6-phosphate or PP_i is located on the cytoplasmic side while that for the glucose acceptor is on the luminal side of the endoplasmic reticulum. Results with neonatal animals indicate a high degree of latency for the phosphotransferase soon after birth, quantitatively altogether comparable with the latency seen in the adult animal. In contrast, the latency of the hydrolase activity was appreciably greater in the neonatal liver than in the adult. The percentage of glucose-6-phosphatase activity that was latent reached a peak at about three to five days of age and corresponded in time with the maximum quantity of postnatal "overshoot" in absolute enzyme activity in rough membranes.

The study is being extended to include the determination of a number of other enzymes that are known to be characteristic of microsomal membranes. Preliminary results show that both nucleoside diphosphatase, known to occur

on the luminal side of the membrane, and NADPH-cytochrome-c reductase, located on the cytoplasmic side, exhibit the same pattern of development with age for the smooth as for the rough membrane fractions. This development differs greatly from that of glucose-6-phosphatase. The results are compatible with the assumption that glucose 6-phosphatase is synthesized in situ on bound ribosomes and then transferred laterally or by ribosome removal to smooth membranes, while the other two enzymes may be made on free ribosomes and subsequently transferred to both rough and smooth membranes.

These studies will be continued and further extended to include S-palmitoyl-coenzyme A-glycerol-3-P acyltransferase, a membrane enzyme important in the synthesis of the phospholipid components of the membranes.

In a related study, carried out in part at the Marine Biological Laboratory at Woods Hole, Massachusetts, we are investigating particulate enzymes of the organs of marine invertebrates. The horseshoe crab, Limulus polyphemus has been found to have an enzyme which resemble vertebrate glucose 6-phosphatase in its specific anatomical distribution, pH optimum, kinetic properties, phosphotransferase activity, substrate specificity and phospholipid dependence. A variety of other invertebrates tested exhibited little or no enzyme activity with these properties. The hypothesis that a specific glucose-6-phosphatase is to be found only in those animals which utilize free glucose as an important circulating form of energy has been proposed. It appears that a variety of transport compounds, such as trehalose and glucose, was tried at the evolutionary level of Arthropods. These studies will be extended to include other invertebrate animals and a variety of enzymes of carbohydrate and lipid metabolism.

Publications:

None.

ANNUAL REPORT OF THE LABORATORY OF CHEMISTRY

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

SECTION ON BIOCHEMICAL MECHANISMS

Chemistry of Imidazoles

The adjacent lone pair (ALP) effect has now been covered in detail. It has been shown that the sp^2 lone pair on a ring nitrogen prevents the generation of a carbanion (and isotope exchange) at the adjacent C-4 position, but less so at C-2. Hammett correlations between the electronegativity of the substituent and the ease of carbanion formation have been demonstrated. A new mechanism for acid-catalyzed exchange, and the sensitivity of all exchanges to buffer catalysis have been discovered. The new ALP theory accounts for a number of unusual imidazole reactions which have previously defied explanation. The theory is applicable to all nitrogen heterocycles, and points the way to practical techniques for selective isotopic labeling of such heterocycles, as well as to the introduction of a variety of substituents under mild conditions. The chemistry of imidazole cyclic trimers has been explored further. Isotopic exchange behavior, as well as various physical and chemical properties, have been determined. It is now clear that these trimers constitute the first heteroannular systems in which the ring current is compelled to pass through the heteroatoms. The possibilities of selective binding of such trimers to DNA and RNA are being investigated.

In the course of preparation of 4,5-difluoroimidazole riboside, it has been found that the reactivity of the fluorine atom toward displacement is much reduced relative to that of 4,5-difluoroimidazole itself. This difference in behavior is puzzling and is under investigation. After considerable effort, the 2-fluoroimidazole-5-carboxamide series has been achieved in a six-step synthesis. The obstacles encountered in this synthesis emphasize the nonaromatic character of 2-aminoimidazoles. These compounds are now being converted into ribosides for antiviral testing.

The biochemical properties of fluoroimidazoles suggest that cyanoimidazoles may also be active toward enzymes and receptors. Initial efforts to develop a general method for the introduction of the cyano group have been successful. Irradiation of 4-nitroimidazoles in acetonitrile containing quaternary ammonium cyanides results in direct replacement of the nitro group by cyano. The possibility of synthesis of 2-cyanoimidazoles by reaction of N-alkylimidazoles with cyanogen is also being explored. (J. Reepmeyer, Y. Takeuchi, R. Jerussi, K. L. Kirk, and L. A. Cohen)

Antiviral Activities of Fluoroimidazoles

New fluoroimidazoles have been synthesized for testing as antiviral agents and inhibitors of polynucleotide biosynthesis. Most recently, the 2-fluoro-4-carboxamide series has become available, while the 2-fluoro-4-amino-5-carboxamide series is still synthetically recalcitrant. Animal testing with FICAR is in progress at Chemie Grünenthal (Germany). The ability of 2-fluorohistidine to serve as an antiviral agent has been demonstrated, activity being based on its ability to substitute for histidine in protein and enzyme biosynthesis in the host cell. Accordingly, false enzymes and viral coat protein are formed, and prevent viral multiplication. (E. De Clercq, J. C. Reepmeyer, K. L. Kirk, and L. A. Cohen)

Hormones and Hormone-releasing Factors

The observation of 20-30% activity in 2-fluorohistidine analogs of THRF and LHRF has prompted a study of the solution conformation of these analogs. Since the basicity of the imidazole ring has been reduced from 7 to 2 in the fluoro analog, it would seem that imidazole basicity cannot be a significant factor in binding to a receptor. The alternative is recognition of the overall conformation of the peptide, as determined by functional groups. Conformational studies are based on ^{13}C nmr spectra of the peptides and their fluoro analogs. A technique has been developed for the replacement of N-terminal histidine in glucagon by fluorohistidine (M. Lin and M. Rodbell); since the activity of glucagon seems to depend critically on the presence of this histidine residue, the properties of histidine analogs may provide essential information on the basis of activity of glucagon. (M. Monahan (La Jolla), H. Yeh, K. L. Kirk, and L. A. Cohen)

A series of ring-halogenated histamines have been prepared and evaluated in an H-1 receptor system (gastric secretion). Of the compounds assayed, 4-chlorohistamine was nearly as effective as histamine in causing HCl secretion. Surprisingly, 2-fluorohistamine was considerably less potent, but has been shown to be recognized selectively by H-2 receptors. (M. Grossman K. L. Kirk, and L. A. Cohen)

Fluorohistidines in Protein Biosynthesis

Studies are continuing on the incorporation of fluorohistidines into animal and bacterial protein. It has now been shown that 4-fluorohistidine is not incorporated to any detectable level. The possibility is being investigated that 2-fluorohistidine is incorporated more rapidly into some proteins and enzymes than into others. Further, it is possible that some fluorohistidine-containing enzymes have not lost activity; this method of study can reveal enzymes in which histidine is not essential to binding or activity. The incorporation of 2-fluorohistidine into newly synthesized hemoglobin has been observed, and studies are continuing on preparation of a quantity of this material (A. Lapidot, Israel).

Halogenated Phenolic Amines in Biochemistry and Pharmacology

A series of ring-fluorinated tyramines, dopamines, and 5-hydroxytryptamines have been synthesized and evaluated in various biological systems. Difluorotyramine is a selective MAO inhibitor: tyramine is bound and metabolized by both A and B types of MAP; 3,5-difluorotyramine behaves like a normal substrate toward the A enzyme, but bonds strongly and reversibly to the B enzyme without being metabolized. This unexpected difference in behavior has led to a working hypothesis to explain structural requirements for the two types of enzyme, and should prove valuable in additional biochemical and clinical studies (K. L. Kirk, D. Murphy, C. Donneley). It has been found that tyramine, 3-fluorotyramine, and 3,5-difluorotyramine displace serotonin from human platelet storage vesicles. While the degree of uptake decreases as the acidity of the phenolic OH increases (with increasing fluorine substitution), the ratio of serotonin displaced per molecule of amine taken up increases in the same order, with a displacement ratio of 400/1 observed for 3,5-difluorotyramine. The significance of these displacement ratios to the internal structure of the storage granule is being investigated. An analogous study with the fluorinated analogs of serotonin is in progress (J. Costa, K. L. Kirk). A series of ring-halogenated α-methyl-meta-tyramines has been prepared for use as possible γ-scanning agents (K. L. Kirk, I. Kopin, and J. Sun).

The effects of ring-fluorinated dopamines, tyramines, and serotonins on the uptake and release of norepinephrine from mouse atria has been studied (C. R. Creveling, K. L. Kirk). Ring-fluorinated dopamines are also methylated by COMT (C. R. Creveling, K. L. Kirk). The effects of ring-fluorinated serotonins on several enzymes involved in the metabolism of serotonin are being studied. These include MAO (D. Murphy, K. Kirk), serotonin O-methyl-transferase and N-acetyltransferase (M. Beaven, K. L. Kirk).

Ring-fluorinated N-acetyl-O-methylserotonins (melatonins) have been prepared. Melatonin has been implicated as an anti-ovulation agent. The fluoro analogs have been found to be as much as 10x as effective as melatonin in organ studies (D. Klein, K. L. Kirk).

Stereopopulation Control and Models for Enzyme Action

We have argued that the deformation of a substrate and consequent activation, in the course of its binding to an enzyme, may contribute far more to the overall rate enhancement phenomenon (enzyme catalysis) than has previously been supposed. In an effort to support the theory, we have synthesized and studied an extensive series of intramolecular systems in which the reacting groups are frozen into van der Waals proximity and optimal orientation by appropriate substitution. In the past year, kinetic studies have been completed on a series of o-hydroxyphenyl-acetic acids. It has been found that, while rate enhancement is somewhat less than in the six-membered lactone series, the difference cannot be attributed to greater ring strain, but to a less effective orientation in the ground state. The synthesis of o-mercaptophenylpropionic acids has achieved after considerable effort, and kinetic studies are in progress.

These models were designed to simulate the function of sulfhydryl group enzymes, such as papain, and to show that the ability of the sulfhydryl group to cleave a peptide bond and generate a high energy thiol ester is wholly consistent with our concepts of substrate activation. Fluorinated analogs of several of our rate-enhancement models have been synthesized. Nmr studies, based on the temperature dependence of fluorine-hydrogen coupling, have revealed a high energy barrier to conformational freedom, ruling out steric hindrance as a basis for restriction. This is the first successful effort in the use of fluorine as a probe for solution conformation, and opens the way for extensive development of this nmr method as a general tool in the study of solution conformation of flexible molecules. (L. A. Cohen, P. S. Hillery, M. Blum, and M. King).

SECTION ON CARBOHYDRATES

Immunoglobulin Work

Subunit Interactions

Heterologous recombinants of H and L chains of homogeneous immunoglobulins with antigalactan activity have been prepared. The overall conclusions are: (1) The idiotype of the recombinant is mostly related to the donor of the heavy chain. (2) The altered fluorescence on ligand binding is also related mostly to that of the H-chain donor. (3) It is possible to prepare a recombinant antibody with higher binding affinity for ligand than the natural H and L donors. (4) Most recombinants form H_2L_2 units, but not all and in one case the H_2L_2 fraction contained H-trimer. (5) All heterologous recombinants can be shown to possess two functional binding sites (by binding radioactive galactotetraose).

Work is under way to make heterologous recombinants of H and L chain from two different anti-carbohydrate specificities. (Manjula and Glaudemans)

Antifructan Myeloma Immunoglobulins

A general method to link polysaccharides to sepharose has been worked out and used to prepare grass-levan- and inulin-sepharose columns for the affinity chromatography of anti-inulin and anti-levan immunoglobulin (Tomasic and Glaudemans). The grass levan in question is being examined chemically, and Hakamori methylation has generally shown that it is a 2→6 linked levan (Tomasic). Fractionation of one myeloma immunoglobulin AB-48 suggests that it may contain two different immunoglobulins. This is being pursued. (Tomasic)

A number of ligands have been prepared which chemically mimmick part of the (2→1) linked or (2→6) linked fructan chain. Some of these are being used to study their binding to immunoglobulins and it appears that a direct correlation can be found between antigenic exposed structure and the capability on the antibody to interact with that exposed structure. (Streefkerk, Ness and Glaudemans)

The synthesis of related carbohydrate ligands for evaluation with anti-

fructan immunoglobulins is underway. (Ness and Das)

Antigens Arising in Virus-transformed Cells.

It appears that the sulphated carbohydrated moities in glycoproteins of virus-transformed cells may be important in antigenic determination. No method for the structural location of sulphate groups on polysaccharides is known to date. A method which shows promise is being developed. (Orme and Glaudemans)

Affinity Labeling of Immunoglobulins

A β-(α-keto diazo) derivative of galactopyranoside is being prepared for affinity labeling. These keto diazo compounds can be activated to great reactivity by Cu^{++} and light and seem very suitable for controlled reaction in the combining site. (Zissis and Glaudemans)

SECTION ON MEDICINAL CHEMISTRY

Testing for Analgesic Activity, Dependence Liability and Narcotic Antagonism

This project is designed for "base-line" studies for analgesic activity by the hot-plate and Nilsen methods (subcutaneous and oral administration) and acute toxicity for more interesting compounds. These more promising compounds are further assessed for physical dependence and antagonistic properties in Rhesus monkeys. Occasionally self-administration experiments (for psychologic abuse potential) are performed.

Approximately 130 new compounds from the (world-wide) pharmaceutical industry, American universities and the Laboratory of Chemistry have been examined during the past year. Of especial interest has been the discovery of codeine-like analgesic activity in the simple bicyclic terpene, (-)-3-isothujone, the rationale for which was its alleged topological similarity to Δ^9-THC, the active principal of marihuana. Also noteworthy was the activity displayed by some deoxy 1,6-methano-3-benzazocines (benzomorphans are 2,6-methano-3-benzazocines) and 6,6-dialkyl-3-benzazocin-8-ols (E. L. May and E. L. Atwell).

Study of the Effect of Analgesics and their Antagonists on the Narcotic Receptor

Different types of analgesics and narcotic antagonists were examined by a variety of *in vivo* and *in vitro* techniques to discern their effect on the narcotic receptor. For example, heroin, 6-acetylmorphine, 3,6-diacetylnormorphine and 6-acetylnormorphine were tested *in vivo*, were examined on guinea-pig ileum, and their affinity for the receptor in rat brain homogenate was determined in an effort to find the mode of action of heroin on the narcotic receptor *in vivo*. Longer-acting narcotic agonists and antagonists were prepared, as were potential irreversible inhibitors of the narcotic receptor. Some of these compounds were tested using the above-mentioned animals and systems, in monkey species, and in an adenylate cyclase assay. Further, the relatively long-acting narcotic antagonists which were prepared gave insight into the molecular configuration of the narcotic receptor (A. E. Jacobson).

Synthesis and Evaluation of Potential Antiinflammatory, Analgesic and Anticancer Agents.

Synthesis of certain members of new classes of potential anti-inflammatory agents was done in order to find new structural types of compounds with antiinflammatory activity, which lack the undesirable side effects of carboxylic acids. Several compounds synthesized produced phosphodiesterase inhibition *in vitro* equivalent to indomethacin and one showed significant activity in the adjuvant arthritis rat screen. Analgesic activity of the simple nonnitrogenous bicyclic terpene, (-)-3-isothujone was found to be equivalent to codeine in mice while several similar compounds were inactive. This substance also exhibited structural stereospecificity suggestive of a specific receptor interaction and would not support dependence in morphine-addicted monkeys. Synthesis and optical resolution of 2,5-dimethyl-2'-hydroxy and 9α and 9β-propyl-6,7-benzomorphans gave analgesics which showed no physical dependence liability in a monkey species and were narcotic agonists or agonist-antagonists with morphine-like or greater analgesia in mice. Replacement of the N-methyl group in the racemic 9α compound with larger alkyl groups gave long acting narcotic antagonists which were examined by a variety of *in vivo* and *in vitro* methods. Potential anticancer thiosemicarbazones were synthesized. The nature of the conformational preference and the N-O bond in several 1,2-oxazepins and related compounds was studied by ^{13}C NMR (K. C. Rice).

Antiinflammatory Heterocyclics and the Interaction of Dextro-Analgesics with Their Receptor

New heterocyclics based on quinolinethione were synthesized. These compounds with possible antiinflammatory activity will be examined in the rat-adjuvant arthritis assay. Dextro enantiomorphs of various narcotic analgesics and antagonists, e.g., morphine and naloxone are being synthesized to ascertain their interaction with receptors (I. Iijima).

Synthesis of Cyclic Amides of Aryl and Alkyl Substituted Picolinic Acids

(1) A number of cyclic amides of substituted picolinic acids were synthesized by standard methods and evaluated for antinociceptive and anti-inflammatory activity. These compounds were also tested in a phosphodiesterase assay. (2) A series of 2-aryl-3-hydroxythieno(2,3-b) quinoline-1,1-dioxides have been synthesized by base-catalyzed cyclization of the corresponding benzyl 2-(3-carbomethoxyquinolyl)sulfones and evaluated for antinociceptive activity. Certain of these compounds have also been tested in a phosphodiesterase assay.

The required benzyl 2-(3-carbomethoxyquinolyl)sulfones were obtained by oxidation followed by esterification of the appropriate benzyl 2-(3-formylquinolyl)sulfides. The latter were, in turn, available from quinoline, thiophosgene and aryl halides through a three-step sequence. By employing a slightly modified oxidation procedure it is possible to produce benzy-2-(3-carbomethoxyquinolyl)sulfoxides instead of the sulfones (E. A. Harrison, Jr. and K. C. Rice).

Synthesis and Pharmacological Evaluation of Unusual Heterocyclic Compounds based on Physicochemical and Topological Analyses

Novel heterocyclic compounds were formulated based on the interatomic distance between certain substituents known to be present in inteferon-inducing substrates. These compounds will be investigated for their antiviral activity. Other new heterocyclics were prepared based on topological estimates of certain potent antiinflammatory compounds and are under investigation for their antiinflammatory activity in vivo and in vitro. Various thiosemicarbazones were prepared and investigated as anticancer agents. At least one of them showed suffieient antitumor activity, at NCI, to warrant further screening (M. F. Rahman and K. C. Rice).

Nordihydromorphine, a By-product in the N-demethylation of morphine

During N-demethylation of morphine by the Rice method (reaction with excess phenylcarbamoyl chloride and subsequent cleavage of the phenylcarbamoyl groups with hydrazine) a by-product, nordihydromorphine, was formed the yield apparently varying inversely with stirring efficiency in the first step of the reaction. Such a reduction of the allylic double bond could result from oxidation of the large amount of phenol formed in the reaction to o-quinone. However, that same reduction of the double bond was caused by hydrazine was shown when a small amount of dihydronormorphine was detected after hydrazine cleavage (24 hrs) of pure N-carbophenoxynormorphine (E. L. May and K. C. Rice).

SECTION ON METABOLITES

Pharmacologically Active Compounds from Tropical Frogs

Additional quantities of histrionicotoxins and pumiliotoxins have been isolated for determination of structure and for investigation of their unique pharmacological properties. Crystals of the hydrobromide of an alkaloid referred to as HTX-D (molecular weight, 285) have been prepared and are under x-ray analysis. This compound is quite different in structure from the congeneric histrionicotoxins. X-ray analysis of crystals of another alkaloid with a molecular weight of 219 was unsuccessful due to disorder in the crystals. Nuclear magnetic resonance spectroscopy has allowed a tentative structural assignment as a diallyldecahydroquinoline. Efforts to characterize structurally an alkaloid responsible for potent analgesic activity of certain frog skin extracts have been unsuccessful due to limited amount of material. Pharmacologically active substances have been detected in four genera of African frogs, Hyperolius, Xenopus, Hylarana and Phyrnomerus. Alkaloids were not detected in representatives of twelve different genera of African frogs. Skins of the common and brightly colored reed frog, Hyperolius parallelus did appear to contain small amounts of alkaloids with molecular weights ranging from 287 to 353. A potent water-soluble toxin present in skins of Colostethus inguinalis of Panama has not been detected in skins of other representatives of this genus. The profile of some 80 different alkaloids in dendrobatid frogs has proven a useful character for studies on evolutionary relationships in this family of frogs (Daly, Mensah-Dwumah).

Batrachotoxin 20-α-benzoate has been prepared and showed unexpectedly high biological activity being equivalent in toxicity and as a depolarizing agent with batrachotoxin itself. This compound has now been prepared in radioactive form for collaborative studies on binding to garfish neurone-membrane fractions, neuroblastoma cells and mouse diaphragm preparations (Brown, Burgermeister).

Histrionicotoxin is virtually inactive in guinea pig ileum preparations, having no effect on muscle tension and failing to antagonize carbamylcholine-elicited contractures. In atrial preparations, histrionicotoxin also has minimal activity, although slight negative inotropic and chronotropic effects were observed. Pumiliotoxin C is a potent agent in atrial preparations causing a profound and reversible decrease in strength and rate of atrial contractures at a concentration of 1 to 2 μM. Atropine does not prevent the action of pumiliotoxin C. Alkaloids of the pumiliotoxin A-series have by contrast, marked positive inotropic and chronotropic effects on the atria (Mensah-Dwumah).

A simple synthesis of the skeleton of histrionicotoxin has been achieved by Schmidt reaction of alkyl 1-alkyl-2-oxo-1-cyclopentane carboxylates to alkyl 2-alkyl-6-oxo-2-piperidine-carboxylates and subsequent Dieckmann cyclization (Kissing, Witkop).

A 4-step synthesis of racemic Pumiliotoxin C has been accomplished by condensation of the pyrrolidine enamine of 2-carbethoxy-5-methyl-cyclohexanone with 3-amino-1-bromohexane followed by saponification, decarboxylation and stereo-preferred reduction (Habermehl, Andres, Witkop).

The dihydroderivative of the alkaloid adalin present in ladybugs has been synthesized by intramolecular 1,3-dipolar addition from a piperidine nitrone with one n-amyl- and one propenyl side chain in the 2-position (E. Gössinger). Unlike Ψ-tropine or β-granatoline this compound has affinity for the muscarinic receptor of neuroblastoma cells (W. Burgermeister).

Ultrastructural characterization of the toxin-glands of frog skin has been initiated. Vesicular entities have been noted. Attempts to isolate such vesicles and demonstrate the presence of alkaloids have not been successful (Creveling, Tice).

Photocyclizations

Photocyclization of benzo[b]thiophene-2-carboxy-N-methylanilide yielded 1-benzothiophene[2,3-c]-trans-14,15-dihydro-5-methylquinolin-6-one, while the lower homologous anilide gave 1-benzothiophene[2,3-c]-cis-14,15-dihydroquinolin-6-one by two distinct mechanisms. The structures were determined by single-crystal Roentgen-ray analysis (Witkop, Yonemitsu, Kanaoka, Iwakuma, Kanamaru, Kimura & Karle).

The Synthesis, Chemistry and Biological Activities of Nucleic Acids and Their Constituents. Interferon Induction by Synthetic Polynucleotides

A number of what we term "hypermodified" C-5 substituted pyrimidine

nucleosides have been synthesized in order to evaluate the effects of the pyrimidine C-5 side chain's steric bulk and stereochemistry on antiviral activity and short-term and long-term toxicity effects. The most interesting of these analogs is when R'=H and R=$CH_2C{\equiv}CH$. This 5-propyryloxy-2'-deoxyuridine has, in tissue culture (primary rabbit kidney cells and human skin fibroblasts), a very substantial activity against herpes simplex-type 1, activity exceeding that of ara-a and approaching that of 5-iodo-2'-deoxyuridine. It is also much less toxic than iododeoxyuridine as judged by its virtual lack of effect on L-cell growth and effect on incorporation of labeled d7 into DNA of stationary PRK cells. From tissue culture studies, we can assign the following anti-herpes inducers (rate of inhibiting $dose_{50}$/inhibitory $dose_{50}$ of viral CPE).

Ara-C	1.5
Ara-A	12.5
5-Iodo-2'-deoxyuridine	12.5
5-Bromo-2'-deoxyruidine	< 5
5-trifluoromethyl-2'-deoxyuridine	125
5-propyryloxy-2'-deoxyuridine	187

5-Propyryloxy-2'-deoxyuridine has several other attributes that make it interesting as a potential antiviral. It is less mutagenic (by a factor of 100) than 5-bromo-2'-deoxyuridine. It does not activate (in BALB/373 cells) expression of oncornavirus under conditions where iododeoxyuridine and bromodeoxyuridine do so.

5-Cyano-2'-deoxyuridine has been synthesized and found to have substantial activity against vaccinia virus. Thus far its apparent lack of toxicity is also remarkable. The anti-vaccinia index for 5-cyano-2'-deoxyuridine has been determined as > 100, compared to 125 for ara-A and 6 for iododeoxyuridine.

A new route to nitration of pyrimidine bases and nucleotides has been found. This provides a convenient synthesis for nitropyrimidine nucleosides and nucleotides for evaluation as precursors to synthetic nucleic acids or as antiviral agents.

Circular dichroism studies have revealed conformational differences between poly(c^7A) duplexes, inactive as interferon inducers, and poly(c^7I) duplexes, active as interferon inducers.

5-Thiocyanato-2'-deoxyuridine and 5-Thiocysnatouridine have been shown to possess *in vitro* antiparasite activity against *Brugea pahangi* and *Schistosoma mansoni*. No other pyrimidine nucleoside, including FUDR, IUDR, F_3TdR shows any activity against these parasites.

Analgesics Based on the Piperidine Ring System

Several compounds exhibited potency in the codeine-morphine range in the mouse hot-plate assay. Two piperidinol esters showed no morphine-like physical dependence capacity in monkeys. Typical narcotic agonist or antagonist activities were absent (Waters, Polansky).

Synthesis of Nucleoside Analogs with Possible Antiviral Properties

Synthetic routes to alanosine (N_β-hydroxy-N_β-nitroso-2,3-diaminopropionic acid) derivatives suitable for a projected synthesis of a modified adenosine nucleotide have been developed which feature novel isoxazolidine-5-one intermediates. The alanosine-modified nucleotide is anticipated to exhibit antiviral properties (Spande, Torrence).

Synthesis and Elucidation of Natural Products

A three step synthesis of a potent reverse transcriptase inhibitor analogous to gliotoxin has been developed in collaboration with H. C. J. Ottenheijm (Nijmegen, Holland) (Spande).

Elucidation of Batrachotoxin "Dimer" Structure

A proposal for the structure of batrachotoxin and homobatrachotoxin "dimers" is being tested in collaboration with T. Tokuyama (Osaka, Japan) (Spande).

SECTION ON MICROANALYTICAL SERVICES AND INSTRUMENTATION

Service Function and Instrumentation

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 government agencies. Approximately 30 of the more common elements and five functional groups are 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 when requested. Instrumental analyses include: GC/MS spectrometry, gas-liquid chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, atomic absorption spectrophotometry, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request (David F. Johnson).

Characterization of Steroid Transforming Enzymes in Tetrahymena Pyriformis

The recent isolation of 4-androstene-3β,17β-diol as a transformation product from incubation T. pyriformis with testosterone in this laboratory indicates that this protozoan has a dehydrogenase capable of reducing the 3-ketone of a steroid to a 3β-hydroxyl. Our objective is to purify and characterize the nature of this enzyme. The identification of several other transformation products is also being pursued in the hope of establishing that T. pyriformis has an isomerase that can transform a Δ^4-steroid to a Δ^5-steroid (Nancy S. Lamontagne).

Application of NMR in Biochemical and Biological Systems

The purpose of this project is to develope nuclear magnetic resonance methods for elucidating molecular structure and for studying the interactions within and between molecules, especially those of biological interests (Herman J. C. Yeh).

Development of Chemical Methods and Compounds for the Study of Bioliogy and Medical Problems

The primary goal of this work is to contribute to the investigation and solution of basic biological and medical problems. This is to be done by the development of chemical methods and reagents for the study of the primary, secondary, tertiary, and quaternary structures of biomacromolecules, for the study of the structure and properties of organelles and cell components, and for the selective modification of biomacromolecules; by the study of intra- and intermolecular interactions; and by the study, development, and application of organic and photochemical reactions. Areas of special interest at present are photocyclization, photorearrangements, and sellenium chemistry (Calvin M. Foltz).

Nature of Steroid-Receptor Interactions

The purpose of this project is to define the preliminary steps in steroid hormone action. In particular, a line of glucocorticord responsive rat hepatoma tissue cluture cells will be used to look at: 1) steroid-receptor binding site interactions; 2) effects of steroid binding on receptor conformation; 3) the nature of "activation" of receptor-steroid complexes, and 4) the nuclear binding of activated receptor receptor-steroid complexes (S. S. Simons, Jr.).

SECTION ON OXIDATION MECHANISMS

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

In order to pursue the metabolism, toxicity, and carcinogenicity of hazardous substances, it has been necessary to synthesize and study the properties of a wide variety of potential metabolites. Previous reports have described methods for the synthesis of K-region and non-K-region arene oxides of polycyclic aromatic hydrocarbons. Binding of these arene oxides to nucleic acid and poly(G), potential macromolecular targets for mutagenesis and carcinogenesis has been studied. Both uv spectra and fluorescence have been used to estimate the extent of covalent interaction. The major conclusions of this study are that the size of the hydrocarbon and the type of arene oxide are very important factors. Stable K-region arene oxides bind more extensively than non-K-region arene oxides. The extent of this binding is directly related to the size of the hydrocarbon, possibly due to intercalation, but is not necessarily related to the carcinogenicity of the parent hydrocarbon. (Dansette and Yagi).

A major effort aimed at the synthesis of all twelve of the possible phenolic metabolites has now been completed. The general procedure for the

final step in these syntheses has been either the dehydrogenation of ketones or Friedel-Craft cyclization of aryl acetic acids. In most cases, numerous prior synthetic steps were required to reach the actual precursors of these phenols. One year carcinogenicity tests on mouse skin are in progress with these compounds. (Yagi, Hernandez, Holder, Yeh, Dansette).

Since bifunctional chemical agents which are capable of cross-linking nucleic acid and protein are known to have toxic and carcinogenic activity, a synthesis of a bis-arene oxide was devised. In this procedure, both K-regions of pyrene were cleaved to dialdehydes and then cyclized to epoxides. The resultant 4,5:9,10-bis-oxide of pyrene was found to be inactive as a mutagen and no more than weakly toxic. (Moriarity and Dansette).

Preparation of the long sought after 7,8-diol-9,10-epoxide stereoisomers of benzo[a]pyrene has finally been achieved. The trans 7,8-dihydrodiol can be directly epoxidized to the diol epoxide in which the 7-hydroxy group and the epoxide are on opposite faces of the molecule. Cyclization of a halohydrin derived from the diol produces the other isomer in which the 7-hydroxy group and the epoxide are on the same face of the molecule and can intramolecularly hydrogen bond. This molecular feature accounts for the remarkable 500-fold greater reactivity of this isomer toward simple nucleophiles. Extremely high reactivity (half-life of 30 sec in aqueous media for the more reactive isomer) of these diol-epoxides accounts for the difficulty encountered in their synthesis. (Yagi and Hernandez).

Continued studies on the metabolism of the environmental carcinogen benzo[a]pyrene have shown that the standard fluorimetric assay for enzymatic oxidation of this compound greatly underestimates the amount of phenolic products formed. Determination of the fluorescence yield of metabolites in acid media where the fluorescence of the twelve possible phenols is similar (in contrast to base where they differ by 10^3) indicates a 50% higher turnover number for the system. This study refutes the widely held view that 3-hydroxy-benzo[a]pyrene is the sole if not preponderant phenolic metabolite. (Holder, Yagi, Levin, Lu).

Profiles of metabolites from benzo[a]pyrene, produced by liver microsomes from genetically different strains of mice, have been analyzed with the aid of high pressure liquid chromatography. Previously, these mice had been classed as "responsive" (C57/BL6) or "nonresponsive" (DBA/2J) depending upon whether benzo[a]pyrene hydroxylase was inducible in the liver with 3-methylcholanthrene. They also differ in their susceptibility to carcinogens. The analysis established that i) the C57/BL6 mouse is inducible while the DBA/2J mouse is not even when compared on an individual metabolite level, ii) mouse liver differs from rat liver in that the ratio of epoxide hydrase to the cytochrome P-450 system in the rat is much higher than in the mouse as evidenced by the low levels of dihydrodiols produced by the mouse, and iii) that all primary oxidative metabolites including phenols, quinones, and dihydrodiols are subject to extensive secondary oxidative metabolism under conditions of low substrate concentration, high protein concentration, and long incubation times. Under the latter incubation conditions, substantial amounts (>30%) of metabolites are formed which are no longer extractable into

organic solvent, possibly due to convalent binding to components of the incubation medium. (Levin, Conney, Holder, Yagi).

Reconstitution of the microsomal enzyme system for drug metabolism has taken an important step forward with the purification of epoxide hydrase. The homogeneous preparation was obtained from an ammonium sulfate precipitate of solubilized microsomes after several column chromatographies in the presence of detergents. (Lu, Levin, Daly).

Biological testing of the synthetic benzo[a]pyrene metabolites is now in full progress, and substantial information is presently available. Arene oxides and the corresponding phenols at the 4,5-, 7,8-, and 9,10-positions have been examined for inherent mutagenic activity with bacterial and mammalian cells which lack the capability for drug metabolism.

In both test systems, the six phenols were either weak or inactive. High mutagenic activity was observed for the 4,5- K-region arene oxide while the two non-K-region arene oxides were very weak as mutagens. Carcinogenicity studies on mouse skin showed a different pattern of activity in that the 9,10- and 4,5- oxides were inactive while the 7,8-oxide was a potent carcinogen, although weaker than benzo[a]pyrene. Evidence has been forthcoming which indicated that the 7,8-oxide may not have inherent carcinogenic activity but may be transformed by epoxide hydrase and then the cytochrome P-450 system into the stereoisomers of the 7,8-diol-9,10-epoxide.

The substantial synthetic effort which has been invested in the diol epoxides has proved quite rewarding in that they are among the most mutagenic compounds yet tested with the Chinese hamster V-79 cell line. The stereoisomer with the benzylic hydroxy group on the same face of the molecule as the epoxide ring is 20-fold more mutagenic in the bacterial tests and >40-fold more mutagenic in the mammalian cells when compared to the very mutagenic benzo[a]pyrene 4,5-oxide. Results to this point are highly indicative that the 7,8-diol-9,10-epoxides may be the ultimate carcinogens of the hydrocarbon.

SECTION ON PHARMACODYNAMICS

The Role of Cyclic Nucleotides in the Nervous System

The nature of noradrenergic receptors controlling cyclic AMP-formation in brain tissue has been further characterized. Clonidine, normally considered to be an α-agonist, has been found to interact with cyclic AMP-generating systems at presynaptic and postsynaptic loci in rat cerebral cortical slices. At the postsynaptic loci it blocks the α-adrenergic component of norepinephrine-elicited accumulation of cyclic AMP, and as an α-agonist greatly potentiates the accumulation of cycic AMP-elicited by isoproterenol. Clonidine, in addition, reduces basal levels of cyclic AMP in phenoxybenzamine-treated slices apparently by interacting as an inhibitory agonist at presynaptic α-receptors which control release of norepinephrine. (Skolnick)

Two classes of β-noradrenergic receptors controlling cyclic AMP-generating systems have been detected in cerebellar slices from rats. One β-receptor apparently present in Purkinje neurons is inhibited by β-antagonists and by neuroleptic phenothiazines such as fluphenazine. The other class of β-receptors is apparently associated with neurons of the granule layer and is not inhibited by neuroleptics. This receptor type is absent in cerebellar slices from rats in which the granule neurons were destroyed by neonatal x-irradiation. (Skolnick)

The levorotary isomer of alprenolol, normally considered a very specific β-antagonist and a useful tool for binding studies with β-receptors, is found to inhibit both the α- and β-adrenergic components of (nor)epinephrine-elicited accumulation of cyclic AMP in rat cerebral cortical slices. (Skolnick)

The α-component of norepinephrine-elicited accumulations of cyclic AMP in rat brain slices has been shown to be completely dependent on extracellular calcium ions. In contrast, the β-component of norepinephrine-elicited responses of cyclic AMP-generating systems in brain slices is only partially reduced by the absence of extracellular calcium ions. (Schwabe)

Adenosine deaminase reduces basal levels of cyclic AMP within brain slices, presumably by enzymatic inactivation of "released" adenosine within the slice. Adenosine-antagonists such as theophylline also reduce basal levels presumably by antagonizing adenosine-elicited activation of cyclic AMP-generating systems. In the presence of adenosine deaminase, responses of cyclic AMP-generating systems to amines are markedly reduced, while responses to N^6-phenylisopropyladenosine, a nonsubstrate for the deaminase, are unaffected or somewhat reduced depending on the species. Thus, endogenous adenosine appears important to responsiveness of cyclic AMP-systems in brain slices. This is particularly true in the absence of extracellular calcium. Under such conditions adenosine deaminase completely prevents any response to biogenic amines. (Schwabe)

Norepinephrine, adenosine and a depolarizing agent, veratridine, elicit accumulations of cyclic AMP and cyclic GMP in rodent brain slices. In addition, calcium ions added to cerebellar slices preincubated in calcium-free medium elicits a rapid 2 to 7-fold accumulation of cyclic GMP, while having little or no effect on basal levels of cyclic AMP. The magnitude of the calcium-elicited accumulation of cyclic GMP is dependent on the species used as source of the cerebellar slices and is greatest with guinea pig. Neurotransmitter release did not appear to be involved in either calcium or veratridine-elicited accumulations of cyclic GMP. Veratridine-elicited accumulation of cyclic AMP appears strongly dependent on depolarization-elicited release of adenosine. The calcium-elicited accumulation of cyclic GMP in guinea pig cerebellar slices is not effectively inhibited by many so-called calcium antagonists or potentiated by calcium ionophores. Of the many compounds tested, promethazine is the most potent antagonist of calcium-elicited accumulations of cyclic GMP, with diphenhydramine, brompheniramine, chlorpromazine and imipramine less active. These compounds were only relatively weak inhibitors of cerebellar guanylate cyclases. (Ohga)

Disruption or reduction of noradrenergic transmission in the central nervous system results in rat in a marked compensatory hyperresponsiveness of norepinephrine-sensitive cyclic AMP-generating systems in rat brain slices. Suprisingly depletion of norepinephrine with reserpine, lesions of ascending noradrenergic neurons of the medial forebrain bundle or destruction of noradrenergic terminas with 6-hydroxydopamine has no effect on responses of cyclic AMP-systems to norepinephrine in guinea pig brain, as measured in cerebral cortical slices. The normally highly responsive norepinephrine-sensitive cyclic AMP-generating systems in cortical slices of certain rat strains also do not show adaptive increases after 6-hydroxydopamine. Thus, it appears likely that in certain species and strains of animals a genetically low functional noradrenergic input has already resulted in maximally responsive cyclic AMP-systems. (Creveling, Dismukes, Ghosh, Skolnick)

The responsiveness of cyclic AMP-generating systems has been compared in brain slices of rats reared in enriched or impoverished environments. Alterations in responsiveness might then provide indications of heightened or reduced functional activity of certain neurotransmitter systems as a result of environmental input. Responses to histamine appeared to be higher in "enriched" rats, while responses to prostaglandin were higher in "impoverished" rats. (Dismukes)

Accumulations of cyclic AMP elicited by norepineprhine and adenosine in cerebral slices form eight strains of rat have been ascertained. No active avoidance learning were apparent either between strains or between individuals of an outbred strain. This contrasts to previous data indicating a negative correlation between norepinephrine-elicited accumulations of cyclic AMP in cerebral slices of four rats strains and the spontaneous behavioral activity. (Dismukes, Stalvey)

A new class of phosphodiesterase inhibitors, 4-(3,4-dialkoxyphenyl)2-pyrrolidones, has been found to be extremely potent with regard to potentiation of norepinephrine and adenosine-elicited accumulations of cyclic AMP in brain slices. The pyrrolidone like the less active 3-(3,4-dialkoxybenzyl-imidazolidinones appeared to inhibit rather selectively cyclic AMP and not cyclic GMP-phosphodiesterases. Inhibition of calcium-dependent cyclic AMP-phosphodiesterase activity by the pyrrolidone and the imidazolidinone appeared to correlate with their potency in increasing accumulations of cyclic AMP in brain slices. The pyrrolidones because of the potency and apparent lack of effect on adenosine "release" would appear the agents of choice for study of cyclic AMP-phosphodiesterases in brain tissue. Isobutylmethylxanthine appears the most effective inhibitor of cyclic GMP-phosphodiesterases based on studies with brain slices. (Miyake, Ohga and Schwabe)

A calcium-dependent activator protein has been purified from rat brain to homogeniety and coupled to I) cyanogen bromide-activated Sepharose or II) N-hydroxysuccinimide-activated succinylated aminopropyl agarose. Affinity chromatography with I but not II led to an excellent separation of calcium-dependent phosphodiesterases from rat brain homogenates. (Creveling, Miyake)

Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

The effects of 6-hydroxydopamine, 5,7-dihydroxytryptamine and several related compounds were examined with regard to the mechanism of neurocytotoxicity in model systems. An oxygen-dependent binding and subsequent cross-linking of model proteins was demonstrated. This binding and cross-linking phenomena occurred primarily through free sulfhydryl groups on proteins. Attempts were made to relate this model for cytotoxic action to events in neurobastoma cultures, in isolated mouse atrial preparations and in intact mice. 6-Hydroxydopamine is bound irreversibly to atria both *in vitro* and *in vivo* and results in a deficit in the active transport system for the normal transmitter, norepinephrine. Similar results were obtained with 5,6-dihydroxytryptamine *in vitro*. While the present results indicate a relationship between the irreversible binding of neurotoxic amines and the cytotoxic sequelae, no direct evidence for amine-induced protein-polymerization in isolated atria or *in vivo* has been obtained. (Rotman) A study of the effect of 6-hydroxydopamine and related compounds on the morphology and response to Ca^{++} of the calcitonin-containing cells of mouse thyroid have demonstrated a relationship between sympathetic innervation of the thyroid and the release and regranulation in the calcitonin-containing cell, however, no evidence was obtained for a cytotoxic effect of 6-hydroxydopamine in either thyroid or calcitonin cells. A neurotoxic degeneration of sympathetic neurons in the thyroid followed administration of either 6-hydroxydopamine, 6-hydroxydopamine and 5,7-dihydroxytryptamine. (Creveling)

The cytotoxicity of 5,7-dihydroxytryptamine *in vivo* is blocked by prior inhibition of monoamine oxidase. The possibility that high concentrations of norepinephrine in monoamine oxidase-inhibited animals might block the cytoxicity of 5,7-dihydroxytryptamine through provision of an "electron-sink" which would prevent the accumulation of toxic levels of 'superoxide ion' or hydroxyl ion was investigated. However, animals with lowered endogenous levels of norepinephrine, resulting from either depletion by reserpine or inhibition of synthesis by α-methyl-*p*-tyrosine or both, showed the same sensitivity to 5,7-dihydroxytryptamine and the same protection by monoamine oxidase inhibition. This result supports the binding and cross-linking mechanism for the mechanism of action of cytotoxic amines and suggests that the formation of superoxide radical through oxidation by the amine is not the primary mechanism of cytotoxicity. (Creveling)

A study of the histopathological effect of the dihydroxytryptamine demonstrated that, 1) 6,7-dihydroxytryptamine was acutely toxic (ED_{50}= 30 mg/kg) in mice and that the toxicity was related to ischemic responses in the vaculature leading to necrotic lesions of vessel walls; 2) administration of 5,6-dihydroxytryptamine resulted in a dose and time dependent development of subepicardial lesions of the myocardium which was preceded by a fatty infiltration of myocardial fibers; 3) the remaining dihydroxytryptamines including 5,7-dihydroxytryptamine fail to produce any cardiovascular toxicity and 4) that there is no apparent relationship between the specific neurocytotoxicity and the effects on either the vasculature or the myocardium. (Creveling)

Routine studies on the inhibition of norepinephrine uptake and the release of norepinephrine both *in vivo* and in isolated atrial slices are being continued to find additional compounds which may be useful as investigational tools. The kinetic parameters of the uptake mechanism are currently under study as a model for investigating the mechanism responsible for the transport of amine through the neuronal membrane. An examination of the transport and storage of the fluorotyramines, dopamines and indoleamines in atrial preparations are being examined. The isolated atrial system *in vitro* is being used as a model to measure the release of amines stored intraneuronally. The possible involvement of S-adenosyl-L-methionine-dependent carboxymethyltransferase in the release of amines is being examined through the use of product inhibition of the transferase. (Creveling)

Evidence for the cellular localization of both soluble and "membrane" bound catechol-O-methyltransferase has been obtained through the use of specific antibodies to this enzyme in conjunction with the "PAP" technique and with ferritin-labeled antibody. The enzyme has been tentatively localized in the 'microsomal' or reticuloendothial processes in liver cells. Present studies are directed at a survey of other peripheral sites including the kidney, heart, vasculature and superior cervical ganglia. Localization studies in the brain are progressing more slowly. The antigenicity of the endogenous enzyme in brain is subject to variation with various biochemical treatments and thus may be located in forms which shield the enzyme from antibody attack. (Creveling)

A study of the incorporation of 2-fluorohistidine into mouse protein, *in vivo*, are being continued. The histopathology of 2-fluorohistidine is being examined in adult and young mice. Efforts have been directed towards a demonstration of the incorporation of 2-fluorohistidine in place of histidine in the intact mouse, in organ cultures of rat pineal glands and in globin synthesized *de novo* with a rabbit reticulocyte system. (Kirk, Creveling)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19000-15 LC

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies on the Synthesis of Carbohydrate Derivatives for Biomedical Research

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

PI:

R.K. Ness	Research Chemist	LC NIAMDD

COOPERATING UNITS (if any)

C.P.J. Glaudemans	Chief, Sec. on Carbohydrates	LC NIAMDD
D.G. Streefkerk	Visiting Fellow	LC NIAMDD

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Carbohydrates

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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

The study of methods for synthesizing the β or cis form (C-2 - C-3) of D-fructofuranosides is being continued.

PHS-6040
(12-75)

Project Description:

Work has been temporarily suspended on the attempted synthesis of 9-(β-D-fructofuranosyl)-adenine in deference to the needs of our section for the preparation of three substances desired for binding affinity studies.

Sirupy methyl β-D-fructofuranoside was prepared in large quantities from sucrose and from D-fructose. Methyl β-D-fructofuranoside tetra-p-nitrobenzoate was prepared in crystalline form for the first time. 2'-Methoxyethyl β-D-fructofuranoside (crystalline) was also prepared for the first time. Attempts have been made to prepare 2',2'-bis-(methoxyethyl) β-D-fructofuranoside through a series of reactions beginning with condensation of sucrose, methyl β-D-fructofuranoside, or D-fructose with glycerol or 3-chloro-1,2-propanediol. This work will continue.

Crystalline D-fructofuranose pentabenzoate (not previously described in the literature) has yielded sirupy 1,3,4,6-tetra-O-benzoyl-D-fructofuranosyl bromide which 1) with methanol has yielded methyl α-D-fructofuranoside tetrabenzoate; 2) with acetone-water has yielded the previously known 1,3,4,6-tetra-O-benzoyl-D-fructofuranose; 3) with silver benzoate has formed the previously mentioned D-fructofuranose pentabenzoate; and 4) with silver p-nitrobenzoate has given in crystalline form a new 1,3,4,6-tetra-O-benzoyl-2-O-p-nitrobenzyol-D-fructofuranose from which the bromide should be more easily obtainable in pure form. Characterization work on these compounds is continuing to elucidate their anomeric configuration.

Similar investigations on a crystalline D-fructopyranose pentabenzoate (not previously described in the literature) is in process.

Significance to Biomedical Research and to the Program of the Institute.

Tryptophanyl fluorescence titration has been useful in studying the binding affinities of a group of anti-polysaccharide myeloma proteins having an anti-(1→6)-β-D-galactan activity with a variety of derivatives of D-galactose (Jolley, Glaudemans, Rudikoff, and Potter, Biochemistry, (1974) 13, 3179). In a similar manner it is hoped that a series of β-D-fructofuranosides might be useful in a study on the immunoglobulins with specificities toward either β-D-(2→1)-fructofuranosides or β-D-(2→6)-fructofuranosides (respectively anti-(2→6)-and-(2→1)-B-D-fructans) with the purpose of readily identifying the particular specificity of the protein. One series of three β-D-fructofuranosides, resembling more the aglycon structure of the (2→1)-fructans as one progresses from (a) to (b) to (c), is (a) methyl β-D-fructofuranoside, (b) 2'-methoxyethyl β-D-fructofuranoside, and (c) 2',2'-dimethoxyethyl β-D-fructofuranoside.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19001-04 LC

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Immunochemistry and Reactions of Carbohydrates

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

PI:	C.P.J. Glaudemans	Chief, Sec. on Carbohydrates	LC NIAMDD
Other:	B.N. Manjula	Visiting Associate	LC NIAMDD
	J. Tomasic	Visiting Fellow	LC NIAMDD
	E. Zissis	Research Chemist	LC NIAMDD
	T. Orme	Guest Worker	DCCP NCI
	D. Streefkerk	Visiting Fellow	LC NIAMDD
	M. Das	Visiting Fellow	LC NIAMDD

COOPERATING UNITS (if any)

M. Vrana	NCI
N. Roy	Indian Association for the Cultivation of Science

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Carbohydrates

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	4.5	0

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

How specific are anti-carbohydrate immunoglobulins? What is the nature of the combining site? To answer these questions the interaction of homogeneous immunoglobulins with carbohydrate haptenic groups will be studied. Site mapping will be studied through impact affinity labeling of the same globulins. Hybrid immunoglobulins using H,L recombinants are studied to define subunit interactions in myeloma protein.

PHS-6040
(12-75)

Project Description:

Objectives: Homogeneous immunoglobulins can be obtained from plasma tumors. The study of their interaction with polysaccharide antigens will reveal much about the nature of action of antibodies and about their structure. We are involved in such studies.

Methods Employed: The usual techniques of chemistry and biochemistry are employed, such as affinity chromatography employing hapten-coupled sepharose columns, fluorescence titration, organic synthesis, gas-liquid chromatography, mass spectroscopy, etc.

Major Findings: 1. Anti-levan Immunoglobulins. A polysaccharide extracted from perennial ryegrass showed two components (fraction 1 and fraction 2) by various procedures (borate buffer chromatography on DEAE sephadex, electrophoresis) one of which greatly predominated (fraction 1). Both contained fructose and a very small amount of glucose (presumably from chain termination by sucrose). Methylation of the fractions by the Hakamori method and examination by gas chromatography of the hydrolysis products showed the absence of 3,4,6 tri-O-methyl-D-fructose, indicating both polysaccharides to be 2→6 linked. Coupling of fraction 1, via its N-(2-aminoethyl carbamyl methylated) derivative, to cyanogen bromide treated sepharose has yielded an excellent affinity-chromatography column for the anti 2→6 fructofuranan immunoglobulins. Therefore, there are now available affinity chromatography columns for immunoglobulins with both 2→1 and 2→6 specificity. Grass-levan is the only polysaccharide which (when bound to sepharose) can bind and release anti 2→6 fructan immunoglobulins. Bacterial levan will bind them too tightly, and no method for release has been found yet. (Tomasic and Glaudemans)

2. Purification of Immunoglobulins with Anti 2→6 Levan Specificity. Two ascites, containing proteins with anti-(2→6) levan activity, UPC-10 (IgG) and ABPC-48 (IgA) were submitted to affinity chromatography on a grass levan-sepharose column.

Twenty-five per cent of UPC-10 protein could be eluted with sucrose (0.5 M aq. soln.). The remaining portion could be eluted with 5 M guanidine.

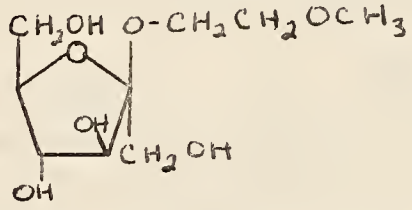
The amount of ABPC-48 protein eluted with sucrose, varied with various batches of ascites, ranging from 25-60%. The remaining portion was eluted with guanidine. Both fractions are IgA proteins, retaining antibody activity after affinity chromatography. Both fractions are high molecular weight polymers. The fraction eluted with sucrose could be reduced to a monomer by mild reduction, while fraction B (eluted with guanidine) remains a polymer after mild reduction.

Heavy and light chains of both A and B fraction were separated on a Sephadex G-100 and compared by isoelectrofocusing. The light chains appeared very similar but not identical. Drastic reductions and alkylation of B yielded molar ratios of H and L chains of 4:1 (OD_{280}), but sequencing of whole B showed the ratio of H/L to be 1 (one amino acid). It is possible that the amount of

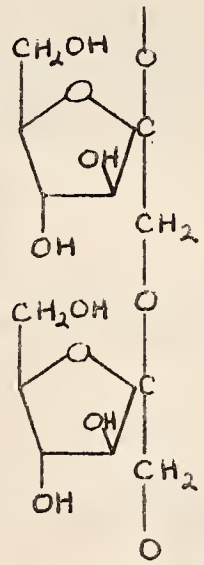
aromatic amino acid in L_B is less than usual. Also, methoxyethyl β-D-fructofuranoside seems to bind to B but not to A (fluorescence). Whether A and B are different immunoglobulins arising from one clone remains to be seen, but it is a distinct possibility. (Tomasic and Glaudemans)

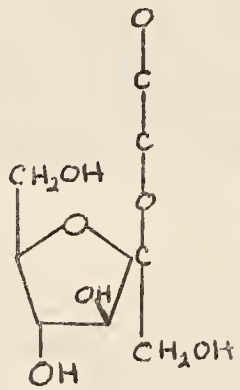
3. Binding Studies of Immunoglobulins with Specificity for (2→6) Fructofuranans. EPC-109, UPC-61, ABPC-4, and ABPC-47 are all immunoglobulins with specificity for (2→6)-β-D-fructofuranans and they precipitate with inulin.

In inulin the chain is terminated by glucose (to form a sucrose residue), and thus inulin is a non-reducing polysaccharide. The binding of the above proteins were studied with ligands resembling in part the structure on inulin. The table (next page) lists the oligosaccharides and myeloma immunoglobulins studied. It can be seen that methoxyethyl-β-D-fructofuranoside



binds not at all, or poorly at best, to the two anti 2→1 fructan binding proteins AB-47 and UPC-61. It is interesting that examination of a molecular model of inulin shows that the carbon atoms indicated below are buried deeply in the antigen, and these carbon atoms would thus not bind well with antibody. It is those carbon atoms which are the aglycon in methyoxyethyl fructofuranoside.





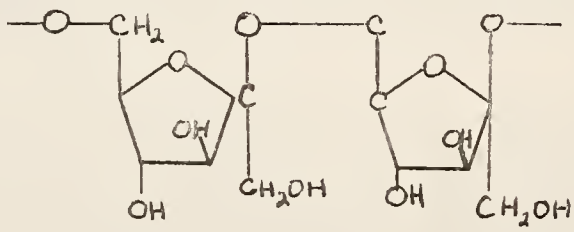
For that reason methyl-β-D-fructofuranoside could also be expected to bind poorly with proteins having anti 2→1 fructan specificity, and this is so (see Table).

Table % quenching of the fluorescence and K_a values of four anti-inulin myeloma proteins with various ligands.[a]

Ligand		EPC-190	ABE-4	ABE-47	UPC-61
Sucrose	%q	7.9	7.5	4.2	4.5
	K_a	880	1040	1040	560
Me-β-D-fructo-furanoside	%q	1.8	3.7	4.7	<1
	K_a	—	—	120	—
Me-α-D-gluco-pyranoside	%q	2.7	2.4	2.1	1.2
	K_a	350	160	180	—
D-Raffinose	%q	5.8	6.7	4.3	4.1
	K_a	270	220	370	(<100)
Methoxyethyl -β-D-fructo-furanoside	%q	a	a	1.3	<1
	K_a			(<100)	—

[a] not yet determined.

Binding with only one anti-(2→6 fructan) immunoglobulin (AB-48) has been tried with the methoxyethyl fructofuranoside. The highly polymeric fraction of AB-48 does show increased fluorescence with the above glycoside. When examining a model of 2→6 linked fructofuranan it seems likely that an antibody could bind with the now exposed oxygen and carbon atoms indicated below. (Streefkerk and Glaudemans)

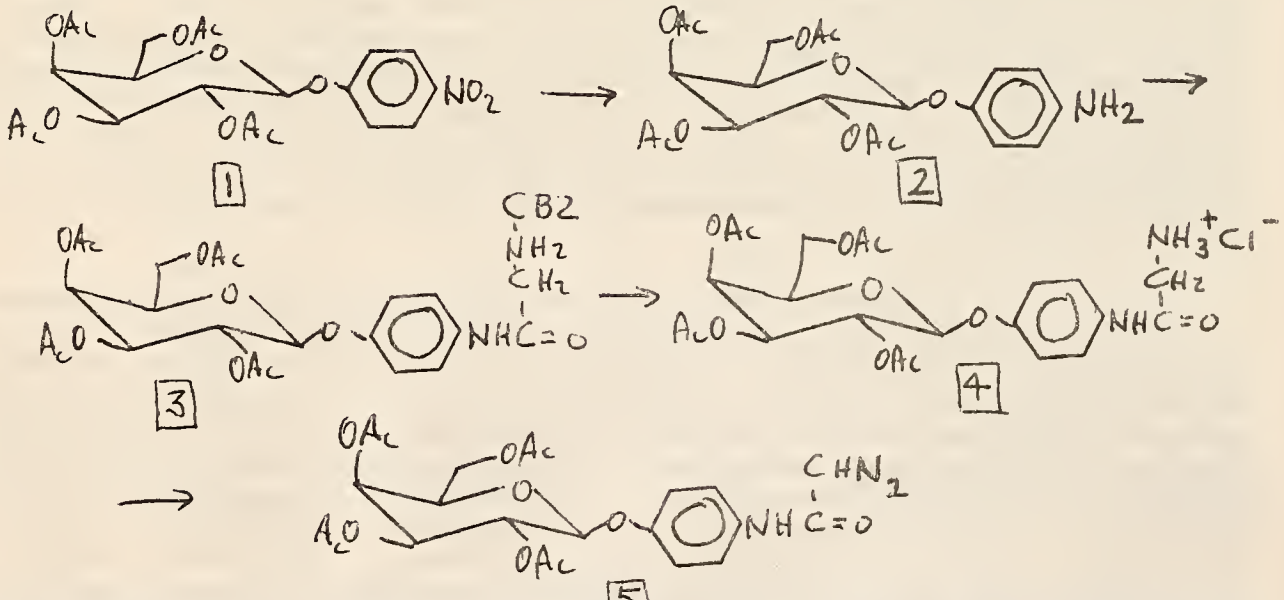


4. Fructose Containing Ligands. Ligands containing fructose or fructose-like carbohydrates are being obtained in two ways: 1) from natural products (graded hydrolysis of inulin, followed by chromatographic separation, D.G. Streefkerk) and 2) by synthesis. Concerning the latter, both R.K. Ness and M. Das are involved in synthetic approaches. The ligands in question are methyoxyethyl-β-D-fructofuranoside (prepared by Ness) and 5-O-β-D-arabino-furanosyl-D-arabinose (Das). The latter compound is identical to levan biose. It is hoped that these ligands will shed light on the exact specificities of the antifructofuranans.

5. Relationships Between Immunoglobulins MOPC-384, MOPC-870 and MOPC-603. The above three Ig's have similar if not identical light chains. MOPC-384 and 870 have the same specificity (lipopolysacc. of S. tranaroa) but MOPC-603 has specificity for phosphorylcholine. We eventually would like to make heterologous H,L recombinations involving these immunoglobulins. We want to obtain pure immunoglobulins by affinity chromatography. We are now involved in preparing S-tranaroa O-antigens to link this to sepharose. (Das and Glaudemans)

6. The Chemistry of Sulphated Sugars. T. Orme (visiting from NCI) is interested in the determinants of antigens on virus-transformed cells. These may involve sulfated sugars. As a first step towards this investigation, a general method is being devised to accurately determine the position of sulphate groups in polysaccharides. (Orme and Glaudemans).

7. Affinity Labeling of Antigalactan Immunoglobulins. The complete synthesis of an α keto diazo galactan derivative as a reactive affinity label is continuing



The last step (4→5) is difficult because the α keto diazo compound 5 is extremely sensitive to acid, and is rapidly lost. (Zissis and Glaudemans)

8. Heterologous Recombinants of S 10 and J 539. Our earlier results on the heterologous recombinants of proteins S 10 (an antigalactan which does not show any significant ligand-induced changes in fluorescence) and X 24 (which shows ligand-induced increase in fluorescence) suggested that in protein X 24, probably the trytophanyl residues in the H chain are responsible for most of the increase in fluorescence observed on ligand binding. These studies have now been extended to another system, namely S 10-J 539 protein pair to see if the same is true for protein J 539.

Heterologous H-L recombinants between S 10 and J 539 were prepared as described in an earlier report. $H^{10}L^{539}$ and $H^{539}L^{10}$ recombinant preparations were prepared as before. The K_a values of $H^{10}L^{539}$ and $H^{539}L^{10}$ recombinant monomers for Gal_2 and Gal_3 are given in Table I, along with the observed increase in fluorescence (ΔF max).

Table I.

Ligand binding characteristics of $H^{10}L^{539}$ and $H^{539}L^{10}$.

Ligand	$H^{10}L^{539}$		$H^{538}L^{10}$	
	K_a	ΔF max	K_a	ΔF max
Gal_2	1.35×10^4	6%	1.68×10^4	6.9%
Gal_3	0.63×10^5	7%	1.63×10^5	6.9%

The ΔF max observed with both the recombinants on binding Gal_2 and Gal_3 was very much lower than that observed with any of the other native antigalactans exhibiting ligand-induced changes in fluorescence. However, the K_a values of both the recombinants compared well with the K_a values for the same ligands with protein J 539 and other native antigalactans. In the case of $H^{10}L^{539}$ the low value of ΔF max may be because H^{10} is not contributing significantly for an increase in fluorescence. J 539 shows a nearly 30% increase in fluorescence on Gal_2 binding. If H^{539} is the predominant contributor to the observed ΔF max of $H^{539}L^{10}$, then the low value suggests that the $H^{539}L^{10}$ preparation might contain some inactive molecules.

Quantitation of the recovery of the binding sites in the recombinants was carried out by determining the amount of radioactive Gal_4 bound to them by the Farr technique. $H^{10}L^{539}$ bound nearly 73% of the theoretical amount of Gal_4 while $H^{539}L^{10}$ bound only 43% of the theoretical amount. It is therefore apparent that the $H^{539}L^{10}$ preparation contains a significant amount of inactive molecules. Idiotypic specificity determinations also indicated the presence of inactive molecules in the $H^{539}L^{10}$ preparation. While $H^{10}L^{539}$ was comparable to S 10 in inhibiting the agglutination of S 10 cells by anti-S 10, $H^{539}L^{10}$ was far less active than J 539 in inhibiting the agglutination of J 539 cells by anti-J 539. The ligand binding activity and the idiotypic specificity of the recombinants therefore suggest that H^{10} and L^{539} combine fairly well to reconstitute native-

like molecules while H^{539} and L^{10} combine poorly, and the $H^{539}L^{10}$ 155,000 MWt fraction contains H-chain trimers.

Analysis of the recombinants for the molar H/L ratio indicated that in $H^{10}L^{539}$ the molar H/L ration was 1.04 whereas in $H^{539}L^{10}$ it was 2.23.

Although the recombinant $H^{10}L^{539}$ gave increase in fluorescence on binding Gal_2 and Gal_3, it showed a decrease in fluorescence with the ligand β-methyl galactoside ($K_a = 0.76 \times 10^3$). Since the change in fluorescence brought about by the two galactose ligands are in different directions, experiments are now being designed to find out if these ligands compete for the same site on the protein or if they bind at different sites on the recombinant.

A similar situation was observed with a native antigalactan protein, CBPC 4. While epoxypropyl-β-galactoside did not bring about any change in fluorescence on addition to the CBPC 4 solution, it could bring down the increase in fluorescence observed on arabinogalactan binding to CBPC 4. These observations are being investigated further.

9. Studies on SAPC 15. SAPC 15 is a mouse myeloma protein which has been shown to precipitate with dextran sulfate. The specificity of the reaction between dextran sulfate and SAPC 15 was established by testing the reaction between dextran sulfate and other myeloma proteins. Four different antigalactans and one antiphosphorylcholine protein (H-8) were tested along with SAPC 15 for reactions with dextran sulfate by ouchterlony double diffusion. None of the antigalactans gave a precipitin line with dextran sulfate. SAPC 15 gave a very strong precipitin line with dextran sulfate. H-8 gave a very weak reaction. The latter is not surprising since it has been shown that the antiphosphorylcholine MCPC 603 can accommodate a sulfate group in its combining site.

SAPC 15 was partially purified by ammonium sulfate fractionation followed by chromatography on Sephadex G 200. This preparation gave a single bond on cellulose acetate microzone electrophoresis. To check if S 15 reacted with sulfated sugar, the partially purified protein was tested in ouchterlony against heparin (a polymer containing glucuronic acid 2-O-sulfate, 2-deoxy-2-sulfoamino-D-glc-6-SO_4, and glucuronic acid), chondroitin sulfate (a polymer containing glucuronic acid and galactosamine 4-SO_4 or galactosamine 6-SO_4) and carrageenan (a polysaccharide containing galactan sulfate). SAPC 15 reacted very strongly with heparin. There was no reaction with chondroitin sulfate and there was a delayed weak reaction with carrageenan. The strong reaction with heparin is suggestive of SAPC 15 being specific to glucose sulfate. The weak cross-reaction with galactose sulfate or the presence of a small number of glucose sulfate residues in the carrageenan sample. It is now proposed that we make an affinity column for the purification of SAPC 15 and study the specificity of this protein in detail. (Manjula and Glaudemans) This project will reinforce Dr. Orms's work on sulfated antigens in virus-transformed cells.

10. Preparation of Benzyl 2,3,4-tri-O-(chloroacetyl)-β-D-glucopyranosiduronic Acid. The synthesis of glycosides usually involved the condensation of a protected glycose derivative with an appropriate aglycon derivative.

Following this, the protecting groups are removed from the glycosyl moiety by treatment with an acid (e.g., isopropylidene groups) or a base (acyl groups), or by catalytic hydrogenolysis (benzyl ethers). The synthesis of 1-O-acyl-glycoses in which the acyl group bears an alkenic bond has long been hampered by the lack of suitably protected glycose intermediates. Earlier work by Bertolini and Glaudemans provided a solution to the problem by the use of the chloroacetyl group, a group that can be removed, at neutral pH, by thiorea in methanol at room temperature, thus avoiding the alkalinity or acidity, or reductive conditions, which would remove 1-O-acyl groups or reduce alkenic linkages. We now report the preparation of benzyl 2,3,4-tri-O-(chloroacetyl)-β-D-glucopyranosiduronic acid, a fairly stable intermediate for the preparation of 1-O-acyl-D-glucopyranuronic acid derivatives where in the 1-O-acyl group is unsaturated.

Benzyl β-D-glucopyranoside was tritylated at O-6 and the resulting ether was chloroacetylated to yield benzyl 2,3,4-tri-O-(chloroacetyl)-6-O-trityl-β-D-glucopyranoside.(1) Detritylation yielded a mixture of (mainly two) compounds from which a crystalline compound having the composition $C_{19}H_{21}Cl_3O_9$ was isolated; this may be benzyl 2,3,6-tri-O-(chloroacetyl)-β-D-glucopyranoside, arising from O-4 → O-6 acyl migration. Consequently, a further portion of (1) was detritylated, and the reaction mixture was immediately treated with potassium permanganate in acetic acid, so that oxidation of the hydroxymethyl group to a carboxyl group would occur without delay. From the resulting mixture, crystalline benzyl 2,3,4-tri-O-(chloroacetyl)-β-D-glucopyranosiduronic acid was isolated. When treated with diazomethane, this acid afforded a crystalline methyl ester. Further work on the preparation of 1-O-acyl derivatives of chloroacetylated glucuronic acid is in progress. (Roy and Glaudemans)

Significance to Biomedical Research and the Program of the Institute:
The Section on Carbohydrates, with its knowledge of immunochemistry and structural carbohydrate chemistry, is in a position to make a real contribution to the study of the immune response, employing these unique sets of immunoglobulins. The specificity of antibodies and the problem of the "economy of the immune response" can be studied very well using this set of immunoglobulins.

Publications:

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Tomasic, J.: Analysis of Glucuronic Acid Conjugates. In Garrett and Hirtz (Ed.) Drug Fate and Metabolism: Methods and Techniques. 1976 in press

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19200-26 LC

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Testing for Analgesic Activity, Dependence Liability and Narcotic Antagonism

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

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Other:	E. L. Atwell	Medical Biology Technician	NIAMDD-LC

COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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

This project is designed for "base-line" studies for analgesic activity by the hot-plate and Nilsen methods (subcutaneous and oral administration) and acute toxicity for more interesting compounds. These more promising compounds are further assessed for physical dependence and antagonistic properties in Rhesus monkeys. Occasionally self-administration experiments (for psychologic abuse potential) are performed.

PHS-6040
(12-75)

Project No. Z01 AM 19200-26 LC

Project Description: This program in collaboration with the National Academy of Sciences, National Research Council, is to examine new compounds for analgesic activity (by the hot-plate and Nilsen methods), acute toxicity, properties of antagonism and abuse liability.

Approximately 130 new compounds from universities, the Laboratory of Chemistry and the (world-wide) pharmaceutical industry were submitted to this program and tested in the hot-plate and Nilsen methods, by the subcutaneous route of administration. Some of the more interesting compounds were tested for oral activity and for acute (24 hr.) toxicity. About 80 compounds were assessed for physical dependence liability and antagonistic property at the Departments of Pharmacology, University of Michigan and Medical College of Virginia. A few were examined for reenforcing (self-administration) properties (psychologic dependence potential).

Of especial interest has been the discovery of codeine-like analgesic activity in the simple non-nitrogenous (-)-3-isothujone, the rationale for which was its alleged topological similarity to Δ^9-tetrahydrocannabinol. Of somewhat lesser note was the analgesic activity displayed by some deoxy 1,6-methano-3-benzazocines (isomeric with 6,7-benzomorphans) and 10-phenyl-decabydroisoquinolines (both new structural types) submitted by the University of Maryland and Dupont, respectively.

Publications:

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Inoue, H. and May, E. L.: Synthesis and pharmacology of 2,9α-dimethyl-2'-hydroxy-6,7-benzomorphan. J. Med. Chem. 19: 259-262, 1976.

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Pert, C. B., Snyder, S. H. and May, E. L.: Opiate recepter interactions of benzomorphans in rat-brain homogenates. J. Pharmacol. Exp. Ther. 196; 316-322, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19201-02

PERIOD COVERED

July 1, 1975 to June 30, 1976.

TITLE OF PROJECT (80 characters or less)

Study of the Effect of Analgesics and their Antagonists on the Narcotic Receptor.

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

PI: A. E. Jacobson Research Chemist NIAMDD-LC

COOPERATING UNITS (if any)

NIMH (W. A. Klee); Medical College of Virginia (M. D. Aceto); University of Michigan (H. H. Swain); Unit for Research on Addictive Drugs, University of Aberdeen (H. W. Kosterlitz).

LAB/BRANCH

Laboratory of Chemistry

SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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

Different types of analgesics and narcotic antagonists were examined by a variety of in vivo and in vitro techniques to discern their effect on the narcotic receptor. For example, heroin, 6-acetylmorphine, 3,6-diacetylnormorphine and 6-acetylnormorphine were tested in vivo, were examined on guinea-pig ileum, and their affinity for the receptor in rat brain homogenate was determined in an effort to find the mode of action of heroin on the narcotic receptor in vivo. Longer-acting narcotic agonists and antagonists were prepared, as were potential irreversible inhibitors of the narcotic receptor. Some of these compounds were tested using the above-mentioned animals and systems, in monkey species, and in an adenylate cyclase assay. Further, the relatively long-acting narcotic antagonists which were prepared gave insight into the molecular configuration of the narcotic receptor.

PHS-6040
(12-75)

Project Description: The antinociceptive activity of analgesics which act through the CNS has occasionally been observed to be directly, and simply, related to their affinity for the narcotic receptor, when the examined series contains structurally similar molecules. Validation was attempted using a different group of analgesics (see K. Rice, Annual Report, for syntheses), the N-substituted 2'-hydroxy-5-methyl-9α-propyl-6,7-benzomorphans.

Major Findings: The 9α and 9β-propyl-6,7-benzomorphans were chosen because a quantitative structure-activity study, using regression analyses, indicated that their biological properties might be of interest.

N-Substituted 2'-hydroxy-2,5,9αtrimethyl-6,7-benzomorphans and the comparably substituted morphines and morphinans exhibit double maxima in antinociceptive activity, at N-methyl and N-amyl or hexyl. However, the comparably substituted 2'-hydroxy-5-methyl-9α-propyl-6,7-benzomorphans do not show this activity profile. The N-amyl and hexyl derivatives in this series were relatively long-acting narcotic antagonists in monkey species. These compounds were examined for antinociceptive activity in several mice screens, and their affinity for the narcotic receptor obtained from rat brain homogenates was determined (by W. Klee, NIMH).

Significance: It is of interest to the Institute to study the basic mechanisms involved in the transmission of pain. The narcotic receptor appears to be part of the sequence of events culminating in pain perception. Our efforts are directed towards elucidating the effects of drugs on this receptor at the molecular level.

Using the 9α-propyl benzomorphans we discovered that a double molecular change was necessary to produce a narcotic antagonist effect in rhesus monkeys. Evidently, with N-amyl and hexyl benzomorphans, the 9α-substituent plays a major role in determining the conformation of the narcotic receptor which leads to the observation of antinociceptive, antagonistic or mixed *in vivo* activities. The known molecular configuration of the substrate gives further insight into the molecular configuration of the receptor required for narcotic antagonist behavior.

Proposed Course: Structurally diverse analgesics will be quantitatively examined, by regression analyses, to relate their *in vivo* antinociceptive activity to their affinity for the narcotic receptor, coupled with other physico-chemical properties.

Publications:

Rice, K. C., Jacobson, A. E. and May, E. L.: Synthesis and analgesic activities of 2,5-dimethyl-2'-hydroxy-9α and β-propyl-6,7-benzomorphans. *J. Med. Chem.* 18: 854-857, 1975.

Jacobson, A. E.: Chemical components of agonism and antagonism. In Snyder, S. H. and Matthysse, S. (Eds.): *Opiate Receptor Mechanisms*, *Neurosciences Res. Prog. Bull.* 13: 61-65, 1975.

Rice, K. C. and Jacobson, A. E.: Preparation and analgesic activity of 3,6-Diacetylnormorphine and 6-acetylnormorphine. J. Med. Chem. 18: 1033-1035, 1975.

Rice, K. C. and Jacobson, A. E.: Optical resolution of (±)-2,5-dimethyl-2'-hydroxy-9α- and 9β-propyl-6,7-benzomorphans and their pharmacological properties. J. Med. Chem. 19: 430-432, 1976.

May, E. L. and Jacobson, A. E.: The chemistry and pharmacology of homologs of 6-acetyl and 3,6-diacetylmorphine. J. Pharm. Sci., in press 1976.

Yeh, H. J. C., Wilson, R. S., Klee, W. A. and Jacobson, A. E.: α- and β-Halomorphides: stereochemistry, analgesic potency, toxicity, and their interaction with narcotic receptors in vitro. J. Pharm. Sci., in press 1976.

Rice, K. C., Lee, W. A., Aceto, M. D., Swain, H. H. and Jacobson, A. E.: Potential long acting opiate antagonists: the preparation, pharmacological activity and opiate-receptor binding of N-substituted 2'-hydroxy-5-methyl-9α-propyl-6,7-benzomorphans. J. Pharm. Sci., in press 1976.

Jacobson, A. E.: Analgesics and their antagonists: structure-activity relationships. In Iversen, L. L., Iversen, S. D. and Snyder, S. H. (Eds.): Handbook of Psychopharmacology. New York, Plenum Publishing Co., Inc., in press 1976.

Jacobson, A. E. and May, E. L.: The chemistry of analgesic drugs. Schoepke, H. G. (Chairman): New York Centennial Symposium on Drugs Affecting the Central Nervous System; Pain, Washington, D. C., Amer. Chem. Soc. (cassette), in press 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 19202-03 LC

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Synthesis and Evaluation of Potential Antiinflammatory, Analgesic and Anticancer Agents

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

PI:	Kenner C. Rice	Staff Fellow	NIAMDD-LC
Other:	M. F. Rahman	Visiting Associate	NIAMDD-LC
	E. H. Harrison	Research Chemist	NIAMDD-LC
	I. Ijima	Visiting Fellow	NIAMDD-LC
	R. Wasylishen	Associate Professor	University of Manitoba, Canada

COOPERATING UNITS (if any)
NIMH (W. Klee); Medical College of Virginia (L. S. Harris, W. L. Dewey, M. E. Rogers, M. D. Aceto); University of Michigan (H. H. Swain).

LAB/BRANCH
Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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

Synthesis of certain members of new classes of potential anti-inflammatory agents was done in order to find new structural types of compounds with antiinflammatory activity, which lack the undesirable side effects of carboxylic acids. Several compounds synthesized produced phosphodiesterase inhibition in vitro equivalent to indomethacin and one showed significant activity in the adjuvant arthritis rat screen. Analgesic activity of the simple nonnitrogenous bicyclic terpene, (-)-3-isothujone was found to be equivalent to codeine in mice while several similar compounds were inactive. This substance also exhibited structural stereospecificity suggestive of a specific receptor interaction and would not support dependence in morphine-addicted monkeys. Synthesis and optical resolution of 2,5-dimethyl-2'-hydroxy and 9α and 9β-propyl-6,7-benzomorphans gave analgesics which showed no physical dependence liability in a monkey species and were narcotic agonists or agonist-antagonists with morphine-like or greater analgesia in mice. Replacement of the N-methyl group in the racemic 9α compound with larger alkyl groups gave long acting narcotic antagonists which were examined by a variety of in vivo and in vitro methods. Potential anticancer thiosemicarbazones were synthesized.

PHS-6040
(12-75)

Project Description: 1. Antiinflammatory Agents. Synthetic studies are proceeding in three areas with the initial objective of finding new non-carboxylic acid structural classes of antiinflammatory agents which lack the undesirable side effects associated with acidic materials. Concurrently, an adjuvant arthritis rat screen is being established to evaluate these compounds in vivo. Activity of several of the target compounds prepared has been determined and initial results indicate that one of them shows significant activity in the adjuvant arthritis screen. Work has begun to examine structural requirements for activity in this series. Inhibition of phosphodiesterase has been suggested as a rapid in vitro screen for compounds with antiinflammatory activity and results obtained for members of the second class of compounds under investigation has shown that several of these inhibit phosphodiesterase to approximately the same degree as indomethacin (a highly active antiinflammatory agent). The in vivo activity of these compounds will be examined. In the area of unusual heterocycles, the chemistry of several previously unknown polycyclic compounds of this type is under investigation and several target compounds have been prepared. These materials will be evaluated in vitro and in vivo in the near future. Continuation of work in the antiinflammatory area is planned along the lines described above in addition to the study of the mechanism of inflammation in vivo.

2. Analgesics. With the objectives of gaining further insight into the nature of the narcotic receptor and elaboration of improved non-addicting strong analgesics, and narcotic antagonists, the preparation and pharmacological evaluation of a number of 6,7-benzomorphans and simple terpene derivatives was undertaken. The synthesis and optical resolution (see K. Rice, Annual Report 1974-1975) of 2,5-dimethyl-2'-hydroxy-9α and 9β-propyl-6,7-benzomorphans provided the (±), (+) and (-) isomers of these compounds and of these, the (±) and (-)-9-α and β isomers showed morphine-like analgesia in the mouse. Examination of the two racemates and their optical isomers for physical dependence capacity (PDC) and narcotic antagonist activity has now been completed using single dose suppression (SDS) studies in morphine dependent monkeys. The (±) 9α compound was shown to have low PDC while the (±) 9β and (-) 9α isomers, both potent analgesics showed neither PDC nor antagonist activity. Most remarkably, the (±)-9β isomer showed no toxic effects (to 16 mg/kg). The (-)-9β isomer, a more potent analgesic than morphine, both an subcutaneous and oral administration showed some antagonist activity and no PDC. The (+) isomers were essentially inactive as expected. The (±)9α and (-) 9β isomers appear to warrant further examination for their potential as non-dependence liable potent analgesics and the (-)-9β compound for its possible utility as an orally effective agonist-antagonist. Synthesis of the N-ethyl through octyl, cyclopropylmethyl and allyl derivatives of the (±) 9α isomer was accomplished by N-demethylation followed by either direct alkylation or an acylation-reduction sequence. (For pharmacology of these compounds, see A. E. Jacobson, Annual Report 1975-1976).

We have previously shown that an unpurified sample of (-)-3-isothujone, a simple, non-nitrogenous, bicyclic terpene has significant analgesic activity in the mouse. We have now prepared pure samples of (-)-3-isothujone

as well as its closely related epimer (+)-3-thujone and found that the former (ED_{50} 6.5) showed analgesic activity equivalent to codeine and no PDC in morphine dependent monkeys. The latter and other similar compounds were essentially inactive in producing mouse analgesia. In addition, racemic 3-isothujone was prepared and found to have approximately one half the activity of the natural (-)-isomer. This combination of both structural and stereospecificity observed for these compounds is suggestive of a specific receptor interaction. Acute toxicities for the 3-thujones were also determined and vastly improved synthetic procedures were developed for two long-known but difficulty accessible 3-thujanols. Additional work is planned in the area of analgesics with the primary goal of further elucidation of the nature of the narcotic receptor and phenomena which result by its interaction with agonist, antagonist and potential irreversible binding agents.

3. Anticancer Agents. Recently a series of 3-thiosemicarbazones were reported in the literature to have significant anticancer activity in several screens. In this work, a torturous synthetic route was utilized to introduce functionality required to solubilize these substances. Because of the activity shown by these compounds and results previously obtained in our laboratory which indicated closely related compounds could be obtained by a greatly simplified procedure, we have prepared a number of candidate drugs which retain several structural features present in the compounds of established activity. In addition, we have prepared several compounds in which the solubilizing group was introduced into a different part of the molecule by simple synthetic manupilations. Results of preliminary biological testing of several compounds appear promising; further work along these lines will be considered when biological evaluation of the series is complete.

Miscellaneous Activities: We have previously shown that the moderately neurotoxic (in mice) 3,3,7,7-tetramethyl-1,2-oxazepin-5-one exists in a single conformation at room temperature (coalesence T = 150°, ΔG for conformational equilibrium = 19.0 ± 0.5 kcol/mole). In order to gain further insight into the nature of the N-O bond and the conformational preference in this and similar compounds, we have examined the influence, on the ^{13}C chemical shifts, of incorporation of a NH grouping, an oxygen atom and the N-O fragment in a saturated seven-membered ring. We have also determined γ and δ long range shielding effects in these systems. Because these long range shielding effects arise primarily through non-bonded interactions, they are very dependent on the three-dimensional structure of a molecule and thus in many cases can provide revealing information concerning the conformation of the molecule under consideration. Additional ^{13}C and ^{15}N NMR experiments are planned which may lead to determination of the exact conformation of certain of these unusually rigid seven membered heterocycles and perhaps also the influence of this rigidity on the biological activity of these compounds.

Selected compounds obtained in the course of the investigations described above have or will be screened for ability to induce production of interferan, for anti-convulsant activity and also antimalarial activity.

Publications:

Rice, K. C., Jacobson, A. E. and May, E. L.: Synthesis and analgesic activities of 2,5-dimethyl-2'-hydroxy-9α- and β-propyl-6,7-benzomorphans. J. Med. Chem. 18: 854-857, 1975.

Rice, K. C.: An improved procedure for the N-demethylation of 6,7-benzomorphans, morphine and codeine. J. Org. Chem. 40: 1850-1851, 1975.

Rice, K. C., Sharpless, N. E., Highet, R. J. and Weiss, U.: Reaction of dimethyl 3-ketoglutarate with 1,2-dicarbonyl compounds III. Exo-Tetracyclo[5.5.1.0.0]tridecane-4,8,12-trione. Tetrahedron Letters 3763-3766, 1975.

Rice, K. C., Weiss, U., Akiyama, T., Highet, R. J., Lee, T. and Silverton, J. V.: Reaction of dimethyl 3-ketoglutarate with 1,2-dicarbonyl compounds IV. Formation of a complex tetracyclic ring-system in aqueous solution at room-temperature. Tetrahedron Letters 3767-3770, 1975.

Rice, K. C. and Jacobson, A. E.: Preparation and analgesic activity of 3,6-diacetylnormorphine and 6-acetylnormorphine. J. Med. Chem. 18: 1033-1035, 1975.

Rice, K. C. and Dyer, J. R.: A practical synthesis of 2,3-dimethylfuran and an efficient stereoselective preparation of Z-3-methyl-2-pentene-1,4-diol. J. Heterocyclic Chem. 12: 1325-1326, 1975.

Rice, K. C. and Jacobson, A. E.: Opitcal resolution of(±)-2,5-dimethyl-2'-hydroxy-9α and β-propyl-6,7-benzomorphans and their pharmacological properties. J. Med. Chem. 19: 430-432, 1976.

Rice, K. C. and Wilson, R. S.: (-)-3-Isothujone: A small non-nitrogenous molecule with antinociceptive activity in mice. J. Med. Chem. in press, 1976.

Rice, K. C., Rubin, Allan B., Boone, B. J. and Rauls, T. J.: Synthesis, antimalarial activity and phototoxicity of some benzo[h]quinoline-4-methanols. J. Med. Chem. in press 1976.

Rice, K. C., Klee, Werner A., Aceto, M.D., Swain, H. H. and Jacobson, A. E.: Potential long acting opiate antagonists: The preparation, pharmacological activity and opiate-receptor binding of N-substituted 2'-hydroxy-5-methyl-9α-propyl-6,7-benzomorphans. J. Pharm. Sci. in press 1976.

Rice, K. C. and Wasyleshen, R. E.: Carbon-13 NMR studies of some saturated 1,2-oxazepin derivatives. Org. Magn. Resonance, in press 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19203-02 LC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Analgesic properties of some terpenoid compounds

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

PI:	Raymond S. Wilson	Research Chemist	NIAMDD, LC
Other:	E. L. May	Scientific Director	NIAMDD, LC
	A. E. Jacobson	Research Chemist	NIAMDD, LC
	K. Rice	Research Chemist	NIAMDD, LC

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

Work temporarily discontinued

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19204-03 LC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Development of cAMP phosphodiesterase inhibitors

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

PI:	Michael E. Rogers	Chemist	NIAMDD, LC
Other:	E. L. May	Chief of Section	NIAMDD, LC
	J. W. Day	Chief Pharmacody.	NIAMDD, LC
	W. Klee,	Biochemist	NIMH,

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

Work temporarily discontinued.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19205-02 LC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Synthesis of 9-methyl-6,7-benzomorphans and their derivatives.

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

PI:	H. Inoue	Chemist	NIAMDD, LC
Other:	E. L. May	Scientist Director	NIAMDD, LC

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

Work temporarily discontinued.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19206-1 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Nordihydromorphine, A By-product in the N-Demethylation of Morphine

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

PI:	Everette L. May	Section Chief	NIAMDD-LC
Other:	Kenner C. Rice	Staff Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

Section on Instrumentation and Analytical Services

LAB/BRANCH

Laboratory of Chemistry

SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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

During N-demethylation of morphine by the Rice method (reaction with excess phenylcarbamoyl chloride and subsequent cleavage of the phenylcarbamoyl groups with hydrazine) a by-product, nordihydromorphine, was formed the yield apparently varying inversely with stirring efficiency in the first step of the reaction. Experiments will be conducted to determine the nature of the reduction involved.

PHS-6040
(12-75)

Project Description: In the Rice N-demethylation of morphine (phenylcarbamate-hydrazine method), in addition to an 80+% yield of normorphine, there is always isolated 10-15% of a by-product with a slightly lower Rf value (silica-gel; $CHCl_3$-MeOH-NH_4OH; 80:18:2). It was noted that the yield of this by-product was greater if the stirring was less efficient in the reaction of phenylcarbamoyl chloride (large excess) with morphine (first step). Although the by-product was never obtained in better than 80% purity (contaminant normorphine) by separation of normorphine through the sulfamate salt, NMR, infrared and chromatographic data in comparison with normorphine and pure nordihydromorphine (prepared by the Rice method) proved that the by-product is indeed nordihydromorphine. It is probably formed through reduction of the allylic double bond in a process involving oxidation of some of the large amount of phenol present to _o_-quinone.

Attempts will be made to determine the mechanism of formation of the nordihydromorphine. A recent experiment indicates some reduction of the allylic double bond by hydrazine.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19207-02 LC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Role of Norepinephrine in the perception of pain

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

PI: L. S. Getsiv
Other: None

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

Work discontinued.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19208-01 LC

PERIOD COVERED
September 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Antiinflammatory Heterocyclics and The Interaction of Dextro-Analgesics with Their Receptors

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

PI:	I. Iijima	Visiting Fellow	NIAMDD-LC
Other:	Kenner Rice	Staff Fellow	NIAMDD-LC

COOPERATING UNITS (if any)
Medical College of Virginia (M. Rogers); University of Michigan (H. H. Swain); NIMH (W. A. Klee).

LAB/BRANCH
Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	0

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

New heterocyclics based on quinolinethione were synthesized. These compounds with possible antiinflammatory activity will be examined in the rat-adjuvant arthritis assay. Dextro enantiomorphs of various narcotic analgesics and antagonists, e.g., morphine and naloxone are being synthesized to ascertain their interaction with receptors.

PHS-6040
(12-75)

Project No. Z01 AM 19208-01 LC

Project Description: 1. A paper has appeared describing that quinoline undergoes ring fission with thiophosgene and base to give o-isothiocyano-cinnamaldehyde, which with base underwent ring closure to 3-formyl-quinoline-2-thione. The presence of two functional groups on quinoline nucleus of 3-formyl quinoline-2-thione suggested that a variety of ring systems could be constructed around the 2 and 3 positons. We have been interested in synthesizing new heterocyclic compounds (see K. Rice Annual Report) to produce antiinflammatory compounds from quinolinethione.

2. It was reported that the dextro enantiomorph of dihydromorphine has proved to be equally as strong an analgesic as morphine. Dextro bases of most narcotics do not possess analgesic properties, but their effect on the respiratory and nervous system may be antagonistic to that of morphine. Further, it has been shown that some dextro benzomorphan enantiomers substitute for morphine in single-dose suppression studies in monkeys, i.e., they may cause physical dependence, perhaps as well as their levo enantiomorphs. These benzomorphans also show little analgesic activity.

The interaction of narcotics with their receptor has been shown to be stereospecific. There is evidence that a separation of pharmacological properties can be accomplished by resolution of these compounds. Further work in this field is being done to see whether other dextro enantiomers can cause physical dependence and, perhaps, use these compounds to find whether there are other receptors in animals specific for the dextro enantiomers.

Major Findings: 1. Some new heterocyclics based on the quinolinethione system have now been synthesized and will undergo testing for antiinflammatory activity in the rat-adjuvant arthritis screen.

2. Considerable progress is being made in the synthesis of the various dextro enantiomorphs of well-known narcotics.

Significance: 1. New antiinflammatory agents which are nonacidic and presumably, would not cause ulcerogenic side-effects would be extremely valuable for the amelioration of the discomfort and, occasionally, the disability, ude to arthritic disease processes.

2. The separation of physical dependence liability, a major side-effect of narcotic analgesics, from analgesia has been a much sought-after goal if a "pure" analgesic is to be found. Thus, the search for different receptors, unlike those known to interact with levo enantiomorphs of narcotic analgesics, may give us information about the biochemical pathways leading to addiction.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19209-01 LC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Unusual Heterocyclics via Physicochemical and Topological Analyses - Synthesis and Evaluation

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

PI:	M. F. Rahman	Visiting Associate	NIAMDD-LC
Other:	K. C. Rice	Staff Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

Medical College of Virginia (M. E. Rogers); National Cancer Institute

LAB/BRANCH

Laboratory of Chemistry

SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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

Novel heterocyclic compounds were formulated based on the interatomic distance between certain substituents known to be present in inteferon-inducing substrates. These compounds will be investigated for their antiviral activity. Other new heterocyclics were prepared based on topological estimates of certain potent antiinflammatory compounds and are under investigation for their antiinflammatory activity in vivo and in vitro. Various thiosemicarbazones were prepared and investigated as anticancer agents. At least one of them showed sufficient antitumor activity, at NCI, to warrant further screening.

PHS-6040
(12-75)

Project No. Z01 AM 19209-01 LC

Project Description: Qualitative and quantitative indicators such as topological analyses and interatomic distances between unique moieties in substrates were used as methods for devising new heterocyclics for synthesis in various medicinal chemistry areas.

The synthesis of various heterocyclic thiosemicarbazones was initiated due to their possible mechanism of action in the coordination of heavy metals by complexation. This might effect the specific transport of these ions across membranes. Thus, these potential antitumor agents were tested at NCI and a few were found to have appreciable activity in initial screens. Further screening of these agents appear warranted by these data. The ion transport phenomena potentially associated with these compounds will be examined by the preparation and antitumor testing of transition metal complexes of these substrates.

Interferon-inducing substrates based on a potentially vital physico-chemical parameter, the interatomic distance between particular moieties in heterocyclic molecules, were synthesized. These compounds will be evaluated for their antiviral activity.

Topological analyses of known antiinflammatory agents indicate direction for the synthesis of various heterocyclic systems. In vivo and in vitro testing of these novel non-acidic heterocyclics is being initiated using several new synthetic reactions discovered in this Section (see K. Rice, Annual Report 1975-1976).

The synthesis and pharmacological evaluation of new agents for the amelioration or alteration of several disease states, based on physico-chemical and topological analyses, is of great importance for these Institutes.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19210-01 LC

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Synthesis of Substituted Picolinamides & 2-Aryl-3-hydroxythieno [2,3-b]-quinoline-1,1-dioxides.

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

PI:	Ernest A. Harrison Jr.	Research Chemist	NIAMDD-LC
Other:	Kenner C. Rice	Staff Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

Medical College of Virginia (M. Rogers)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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

(1) A number of cyclic amides of substituted picolinic acids were synthesized by standard methods and evaluated for antinociceptive and anti-inflammatory activity. These compounds were also tested in a phosphodiesterase assay. (2) A series of 2-aryl-3-hydroxythieno(2,3-b) quinoline-1,1-dioxides have been synthesized by base-catalyzed cyclization of the corresponding benzyl 2-(3-carbomethoxyquinolyl)sulfones and evaluated for antinociceptive activity. Certain of these compounds have also been tested in a phosphodiesterase assay.

The required benzyl 2-(3-carbomethoxyquinolyl)sulfones were obtained by oxidation followed by esterification of the appropriate benzyl 2-(3-formylquinolyl)sulfides. The latter were, in turn, available from quinoline, thiophosgene and aryl halides through a three-step sequence. By employing a slightly modified oxidation procedure it is possible to produce benzyl-2-(3-carbomethoxyquinolyl)sulfoxides instead of the sulfones.

PHS-6040
(12-75)

Project No. Z01 AM 19210-01 LC

Project Description: To synthesize new types of antiinflammatory compounds which have fewer side-effects than those presently known.

(1) A number of cyclic amides of substituted picolinic acids have been synthesized and evaluated for antinociceptive and antiinflammatory activity. In addition, these compounds were tested in a phosphodiesterase assay. The antinociceptive activity was determined in mice using a standard hot-plate assay procedure; the antiinflammatory activity was determined in a rat adjuvant-arthritis assay. From these initial screens, one particular molecule from this series has shown definite antiinflammatory activity.

(2) A series of 2-aryl-3-hydroxythieno[2,3-b]quinoline-1,1-dioxides have been synthesized by methoxide-ion catalyzed cyclization of the corresponding benzyl 2-(3-carbomethoxyquinolyl) sulfones and evaluated for antinociceptive activity. Certain of these compounds have also been tested in a phosphodiesterose assay. The antinociceptive activity was determined in mice using a standard hot-plate assay procedure.

The required benzyl 2-(3-carbomethoxyquinolyl) sulfones were obtained by sodium chlorite oxidation followed by esterification of the appropriate benzyl 2-(3-formylquinolyl) sulfides. The latter were, in turn, available By employing a slightly modified oxidation procedure (i.e., lower temperature and lower sodium chlorite concentration) it is possible to produce benzyl 2-(3-carbomethoxyquinolyl) sulfoxides rather than sulfones.

Results from the phosphodiesterose assay indicate that one particular molecule has activity equal to that of two commonly used antiinflammatory agents (i.e., flufenamic acid and indomethacin).

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19400-11 LC

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Pharmacologically Active Compounds from Tropical Frogs

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

PI:	Bernhard Witkop	Chief, Lab. of Chemistry	LC NIAMDD
	J.W. Daly	Chief, Sec. on Pharmacodynamics	LC NIAMDD
Other:	George Brown	Staff Fellow, Lab. of Chemistry*	LC NIAMDD
	C.R. Creveling	Research Chemistry	LC NIAMDD

* until November 30, 1975

COOPERATING UNITS (if any) C.W. Myers, Am. Museum of Nat. History, N.Y.,N.Y.; E.X. Albuquerque, Dept. Pharmacology, Univ. of N.Y. at Buffalo; I.L. Karle, U.S. Naval Res. Lab., Wash. D.C.; G. Habermehl, Technische Hochschule, Darmstadt, Germany; T. Tokuyama, Osaka City Univ., Osaka, Japan;

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Metabolites

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.5	.5

SUMMARY OF WORK (200 words or less - underline keywords) The structures of diverse toxic alkaloids from frogs of the genera Dendrobates, Colosthetus and Atelopus have been isolated. The pharmacological activity of batrachotoxin and histrionicotoxin analogs have been studied in detail. Radioactive batrachotoxin, tetrodotoxin and histrionicotoxin have been prepared and are being used to study their ultrastructural site of action. The synthesis of dodecahydrohistrionicotoxin, two isomeric decahydrohistrionicotoxins and of octahydrohistrionicotoxin has been accomplished.

PHS-6040
(12-75)

Cooperating Units (Cont'd): L. Tice, Laboratory of Experimental Pathology, NIAMDD; Y. Kishi, Harvard University, Cambridge, Mass.; M. Mensah-Dwumah, Guest Worker, Howard University, Washington, D. C. (Astra Pharmaceutical Company, FAES Grant); W. Burgermeister, Guest Worker, Deutsche Forschungs-Gemeinschaft; George Brown, Center for Development and Learning Disorders, University of Alabama, Birmingham, Alabama.

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 pharmacodynamically active substances found in skin extracts of tropical frogs. To explore biosynthetic pathways involved in the formation of these unique substances.

Methods Employed: Sensitive bioassay methods, thin layer, high pressure liquid, 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. Biological activity has been investigated with both isolated enzyme systems, in intact cells and muscle preparations.

Major Findings: Additional quantitites of histrionicotoxins and pumiliotoxins have been isolated for determination of structure and for investigation of their unique pharmacological properties. Crystals of the hydrobromide of an alkaloid referered to as HTX-D (molecular weight, 285) have been prepared and are under x-ray analysis. This compound is quite different in structure from the congeneric histrionicotoxins. X-ray analysis of crystals of another alkaloid with a molecular weight of 219 was unsuccessful due to disorder in the crystals. Nuclear magnetic resonance spectroscopy has allowed a tentative structural assignment as a diallyldecahydroquinoline. Efforts to structurally characterize an alkaloid responsible for potent analgesic activity of certain frog skin extracts have been unsuccessful due to limited amount of material. Pharmacologically active substances have been detected in four genera of African frogs, Hyperolius, Xenopus, Hylarana and Phyrnomerus. Alkaloids were not detected in representatives of twelve different genera of African frogs. Skins of the common and brightly colored reed frog, Hyperolius parallelus did appear to contain small amounts of alkaloids with molecular weights ranging from 287 to 353. A potent water-soluble toxin present in skins of Colostethus inguinalis of Panama has not been detected in skins of other representatives of this genus. The profile of some 80 different alkaloids in dendrobatid frogs has proven a useful character for studies on evolutionary relationships in this family of frogs (Daly, Mensah-Dwumah).

Batrachotoxin 20-α-benzoate has been prepared and showed unexpectedly high biological activity being equivalent in toxicity and as a depolarizing agent with batrachotoxin itself. This compound has now been prepared in radioactive form for collaborative studies on binding to garfish neurone-

membrane fractions, neuroblastoma cells and mouse diaphragm preparations (Brown, Burgermeister).

Histrionicotoxin is virtually inactive in guinea pig ileum preparations, having no effect on muscle tension and failing to antagonize carbamylcholine-elicited contractures. In atrial preparations, histrionicotoxin also has minimal activity, although slight negative inotropic and chronotropic effects were observed. Pumiliotoxin C is a potent agent in atrial preparations causing a profound and reversible decrease in strength and rate of atrial contractures at a concentration of 1 to 2 ?M. Atropine does not prevent the action of pumiliotoxin C. alkaloids of the pumiliotoxin A-series have, by contrast, marked positive inotropic and chronotropic effects on the atria (Mensah-Dwumah).

Ultrastructural characterization of the toxin-glands of frog skin has been initiated. Vesicular entities have been noted. Attempts to isolate such vesicles and demonstrate the presence of alkaloids have not been successful (Creveling, Tice).

Significance to Biomedical Research and the Program of the Institute: The exceptionally high toxicity of batrachotoxin, its selective action on nerve preparations and its cardiotoxin properties gave the elucidation of its structure and correlations of structures with activity a high priority in biomedical research. Because of their remarkable effects on ion conductances, histrionicotoxin and various analogs provide unique tools for the study of the acetylcholine-modulated ion channels in electrogenic membrane. 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, samandarine, the bufogenins, the catecholamines, indolealkylamines and histamines, and recently hypotensive polypeptides such as bradykinin and physalaemin. The potent analgesic and cardiotonic effects of certain of these compounds are of particular relevance to the design of new drugs.

Proposed Course of Project: The structures of various alkaloids from frogs of the genera Dendrobates, Colosthetus and Phyllobates will be elucidated. The pharmacological activity of batrachotoxin and histrionicotoxin analogs will be studied in detail. Effects of pumiliotoxins on atrial preparations will be studied in detail. Radioactive batrachotoxin, tetrodotoxin and histrionicotoxin will be used to study their ultrastructural site of action. Further isolations of batrachotoxins, histrionicotoxins, and pumiliotoxins will be carried out to provide material for chemical and pharmacological study. Pharmacologically active substances will be investigated in extracts from other families of tropical frogs.

Publications:

Warnick, J. E., Albuquerque, E. X., Lapa, A. J., Daly, J. W. and Witkop, B.: Actions of neurotoxins on the acetylcholine receptor-ionic conductance modulator unit and on sodium channels. Proc. Sixth Inter. Congress of Pharmacol., Vol. 1, 1975, pp. 67-76.

Shotzberger, G. S., Albuquerque, E. X. and Daly, J. W.: The effects of batrachotoxin on cat papillary muscle. J. Pharmacol. Exp. Therap. 196: 433-444, 1976.

Myers, C. W. and Daly, J. W.: A new species of poison frog from Andean Ecuador, including an analysis of its toxins. Bulletin University Kansas Museum Natural History, in press.

Myers, C. W. and Daly, J. W.: Preliminary evaluation of skin toxins and vocalizations in taxonomic and evolutionary studies of the poison-dart frogs (Dendrobatidae). Bulletin American Museum Natural History, in press.

Kissing, W. and Witkop, B.: Ein einfacher Zugang zu 1-Azaspiro[5.5]undecanen. Chem. Ber. 108: 1623-1629, 1975.

Habermehl, G., Andres, H. and Witkop, B.: Synthese von rac.-Pumiliotoxin C. Naturwissenshcaften 62: 345-346, 1975.

APPENDIX

Contracts

Contract No. N01-AM-4-2203; Isolation of Frog Toxins; $6,000; 1 man year.

Extracts of dendrobatid frogs provided by NIH have been fractionated to yield quantities of batrachotoxins and histrionicotoxins for pharmacological evaluation by NIH researchers. Structures of certain new congeneric alkaloids were determined by spectroscopic analysis. Crystals of other more unique alkaloids have been prepared and provided to NIH investigators for X-ray analysis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19401-11-LC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Synthetic Photochemistry

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

PI: Bernhard Witkop Chief, Laboratory of Chemistry LC-NIAMDD

COOPERATING UNITS (if any) O. Yonemitsu, Y. Kanaoka and T. Iwakuma, University of Hokkaido, Sapporo, Japan; N. Kanamaru and K. Kimura, Research Institute of Applied Electricity, Hokkaido University, Sapporo, Japan; I. L. Karle, U.S. Naval Research Lab., Washington, D. C.

LAB/BRANCH
Laboratory of Chemistry

SECTION
Metabolites

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	0

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

The synthesis of novel heterocycles with potential for antiviral and CNS activity has been accomplished by photo-cyclizations and dimerizations.

PHS-6040
(12-75)

Project Description:

Objectives: Reactions for the selective modification of simple amino acids in proteins and of purines and pyrimidines in nucleic acids 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 have been elaborated with a number of homo- and heterocyclic models 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 have been investigated. Studies continue with new heterocyclic systems.

Methods Employed: Ultraviolet irradiation, flash photolysis thin-layer chromatography, silica gel and ion exchange column chromatography, paper chromatography and electrophoresis, UV, IR, NMR and mass spectroscopy.

Major Findings: Photocyclization of benzo[b]thiophene-2-carboxy-N-methylanilide yielded 1-benzothiophene[2,3-c]-cis-14,15-dihydroquinolin-6-one by two distinct mechanisms. The structures were determined by single-crystal Roentgen-ray analysis.

Significance to Biomedical Research and the Program of the Institute: New approaches to novel heterocyclic systems of potential CNS activity.

Proposed Course: Extension into homo- and heterocyclic systems relating to pharmacodynamic amines and to potential antiviral cage compounds.

Publications:

Hirao, K., Taniguchi, M., Iwakuma, T., Yonemitsu, O., Flippen, J. L., Karle, I. L., and Witkop, B.: Stereospecific acid-catalyzed rearrangement of 1,6-dimethylpentacyclo[6.4.0.0.0.0]dodecane-5,12-dione to a Bisnordiadamantane. J. Amer. Chem. Soc. 97: 3249-3250, 1975.

Kanaoka, Y., Itoh, K., Hatanaka, Y., Flippen, J. L., Karle, I. L. and Witkop, B.: Synthetic photochemistry with heterocyclic anilides. Stereochemistry of the intramolecular 1,5-hydrogen shifts in nonoxidative photocyclization of benzo[b]thiophene-2-carboxanilides. J. Org. Chem. 40: 3001-3003, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 19402-03 LC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Interferon Induction by Synthetic Polynucleotides

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

PI:	Paul F. Torrence	Research Chemist	NIAMDD, LC
Other:	B. Witkop	Chief, Lab. of Chemistry	NIAMDD, LC
	B. Bhooshan	Visiting Fellow	NIAMDD, LC
	G. F. Huang	Visiting Fellow	NIAMDD, LC
	A. M. Bobst	Staff Fellow	NIAMDD, LC

COOPERATING UNITS (if any)

Erik De Clercq, Alfons Billian, Rega Institute, Leuven, Belgium; David Stollar, Tufts University; I. Jaffe, University of Vermont.

LAB/BRANCH

Laboratory of Chemistry

SECTION

Metabolites

INSTITUTE AND LOCATION

NIAMDD, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	4	0.5

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

The structural and conformational features that govern interferon induction is investigated by the syntheses and evaluation of new polynucleotides. Various physical (CD) and biological (e.g. antibody) studies are designed to attempt to understand differences between active and non-active inducers. The antiviral, antitumor and antiparasitic activities of novel nucleoside analogs is also under investigation.

PHS-6040
(12-75)

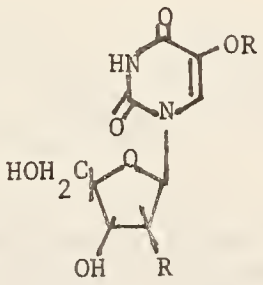
Project Description:

Objectives: To synthesize modified nucleosides, nucleotides and polynucleotides. To characterize such materials by physicochemical means and then investigate the relationship of molecular structure and conformation to certain biochemical and biological activities of nucleic acids. The chief goal of this project is to provide 1) nucleic acid analogs which may be useful in understanding the biochemistry to cytolytic and oncogenic viruses and/or 2) the prophylaxis or therapy of viral diseases. In this latter area, considerable emphasis is currently being placed on the development of an ideal synthetic interferon inducer which may be used in the production of exogenous or endogenous human interferon, and one or more nucleosides that would be useful in the therapy of established viral disease.

Methods Employed: Thin-layer, paper, ion-exchange, gel and other types of column chromatography, liquid scintillation counting, ultracentrifugation, ultraviolet, visible, infrared and nuclear magnetic resonance, spectroscopy, mass spectroscopy, electron spin resonance, circular dichroism, tissue culture.

Major Findings:

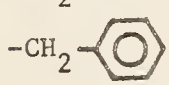
1. A number of what we term "hypermodified" C-5 substituted pyrimidine nucleosides have been synthesized in order to evaluate the effects of the pyrimidine C-5 side chain's steric bulk and stereochemistry on antiviral activity and short-term and long-term toxicity effects.

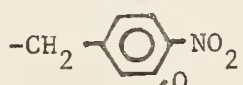


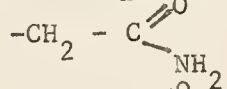
R' = H or OH

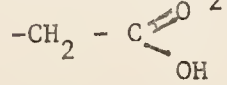
R = $-CH_2-CH=CH_2$

$-CH_2-C\equiv CH$







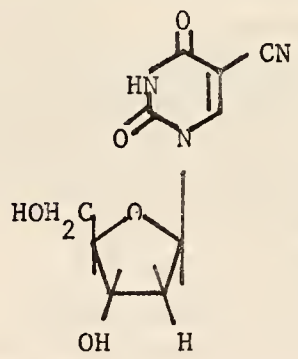


The most interesting of these analogs is when R'=H and R=$CH_2C{\equiv}CH$. This 5-propyryl-oxy-2'-deoxyuridine has, in tissue culture (primary rabbit kidney cells and human skin fibroblasts), a very substantial activity against herpes simplex-type 1, activity exceeding that of Ara-A and approaching that of 5-iodo-2'-deoxyuridine. It is also much less toxic than iododeoxyuridine as judged by its virtual lack of effect on L-cell growth and effect on incorporation of labeled dT into DNA of stationary PRK cells. From tissue culture studies, we can assign the following anti-herpes indices (rate of inhibiting $dose_{50}$/inhibiting $dose_{50}$ of viral CPE).

Ara-C	1.5
Ara-A	12.5
5-Iodo-2'-deoxyuridine	12.5
5-bromo-2'-deoxyuridine	< 5
5-trifluoromethyl-2'-deoxyuridine	125
5-propyryloxy-2'-deoxyuridine	187

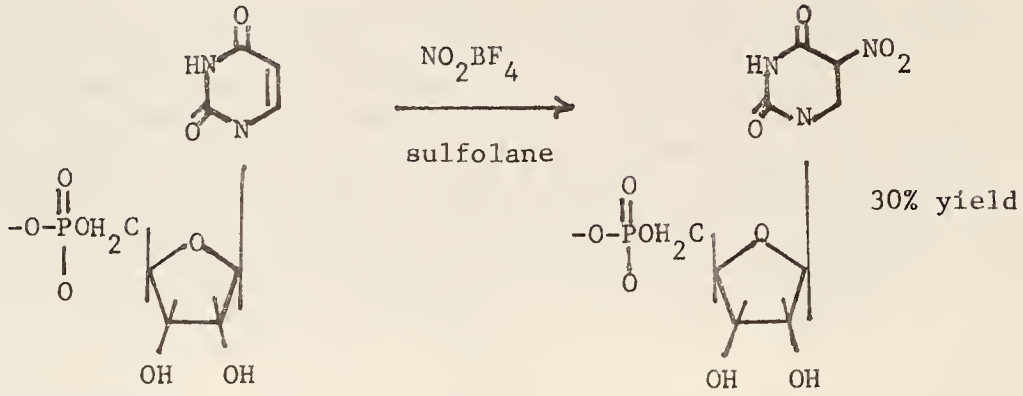
5-Propyryloxy-2'-deoxyuridine has several other attributes that make it interesting as a potential antiviral. It is less mutagenic (by a factor of 100) than 5-bromo-2'-deoxyuridine. It does not activate (in BALB/373 cells) expression of oncornavirus under conditions where iodo-deoxyuridine and bromodeoxyuridine do so.

2. 5-Cyano-2'-deoxyuridine has been synthesized and found to have substantial activity against vaccinia virus.



Thus far its apparent lack of toxicity is also remarkable. The anti-vaccinia index for 5-cyano-2'-deoxyuridine has been determined as > 100 compared to 12.5 for Ara-A and 6 for iododeoxyuridine.

3. A new route to nitration of pyrimidine bases and nucleotides has been found.



This provides a convenient synthesis for nitropyrimidine nucleosides and nucleotides for evaluation as precursors to synthetic nucleic acids or as antiviral agents.

4. Circular dichroism studies have revealed conformational differences between poly(c7A) duplexes, inactive as interferon inducers, and poly(c7I) duplexes, active as interferon inducers.

5. 5-Thiocyanato-2'-deoxyuridine and 5-thiocyanato-uridine have been shown to possess in vitro antiparasite activity against Brugea pahangi and Schistosoma mansoni. No other pyrimidine nucleoside, including FUDR, IUDR, F_3TdR shows any activity against these parasites.

Significance to Biomedical Research and the Program of the Institute: Studies described herein and those in progress are of interest for the following reasons.

1) Modified nucleosides can aid in understanding the biochemistry of viruses; in addition, they are of immediate interest as agents active against viral, and neoplastic disease and, in some instances, as local agents in rheumatoid arthritis.

2) Modified polynucleotides aid in the understanding of protein-nucleic acid interactions and nucleic acid-nucleic acid interactions. These considerations are fundamental for a complete knowledge of cellular biochemistry including on the one hand the regulation of expression of genetic information in eukaryotic cells and, at the more practical level, the induction of interferon and the design of a clinically useful interferon inducer.

Publications:

De Clercq, E., Luczak, M., Shugar, D., Torrence, P. F., Waters, J. A. and Witkop, B.: Effect of cytosine arabinoside, iododeoxyuridine, ethyldeoxyuridine, thiocyanatodeoxyuridine and ribavirin on tail lesion formation in mice infected with Vaccinia virus. Proc. Soc. Exp. Biol. Med. 151: 487-490, 1976.

Torrence, P. F. and Witkop, B.: Polynucleotide Duplexes based on poly(7-deazaadenylic acid). Biochem. Biophys. Acta 395: 56-66, 1975.

De Clercq, E., Torrence, P. F. and Witkop, B.: Polynculeotide displacement reactions: Detection by interferon induction. Biochemistry 15: 717-724, 1976.

Torrence, P. F., De Clercq, E. and Witkop, B.: Triple-helical polynucleotides, mixed triplexes of the polyuridylic acid·polyadenylic acid·polyuridylic acid class. Biochemistry 15: 724-734, 1976.

De Clercq, E., Torrence, P. F., Hobbs, J., Janik, B., De Somer, P. and Witkop, B.: Anticomplement activity of polynucleotides. Biochem. Biophys. Res. Comm. 67: 255-263, 1975.

Johnston, M. I., Stollar, B. D., Torrence, P. F. and Witkop, B.: Structural features of double-stranded polyribonucleotides required for immunological specificity and interferon induction. Proc. Nat. Acad. Sci. U.S.A. 72: 4564-4568, 1975.

De Clercq, E., Torrence, P. F., Witkop, B. and De Somer, P.: Interferon induction by synthetic polynucleotides. Competition between inactive and active inducers. In Geraldes, A. (Ed.): Effects of Interferon on Cells, Viruses and the Immune System. New York, Academic Press, 1975, pp. 215-236.

De Clercq, E. Torrence, P. F., Waters, J. A. and Witkop, B.: Antiviral activity of 5-thiocyanato-pyrimidine nucleosides. Biochem. Pharmacol. 24: 2171-2176, 1976.

De Clercq, E., Billiau, A., Torrence, P. F., Waters, J. A. and Witkop, B.: Antiviral and antimetabolic activities of poly(7-deazaadenylic acid) and poly(7-deazainosinic acid). Biochem. Pharmacol. 24: 2225-2232, 1976.

Torrence, P. F. and Witkop, B.: Design of polynucleotides that act as inducers of interferon. Protein, Nucleic Acid, Enzyme. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19403-03 LC

PERIOD COVERED July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Synthesis and Elucidation of Natural Products

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

PI: Thomas F. Spande Research Chemist LC NIAMDD

COOPERATING UNITS (if any) H.C.J. Ottenheijm, Catholic University, Nijmegen, Holland; T. Tokuyama, Osaka City University, Osaka, Japan; A. N. Radhakrishnan, Christian Medical Hospital, Wellcome Research Unit, Tamil Nadu, India ; Edda Gössinger, Visiting Assoc., Eidgenossische Techische Hochschule, Zurich, Switzerland

LAB/BRANCH
Laboratory of Chemistry

SECTION
Metabolites

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	0

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

To investigate synthetic routes to perhydrohistrionicotoxin, a naturally occurring toxin of the frog Dendrobates histrionicus.

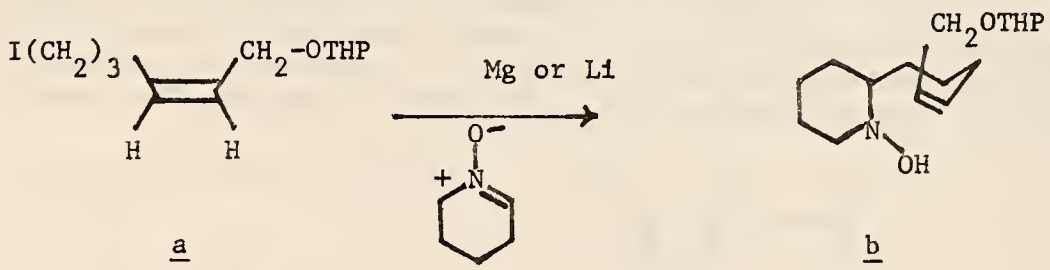
PHS-6040
(12-75)

Project Description:

Objectives: To synthesize analogs of histrionicotoxin, the potent natural frog toxin. To synthesize analogs of gliotoxin. To determine the structures of various naturally occurring substances.

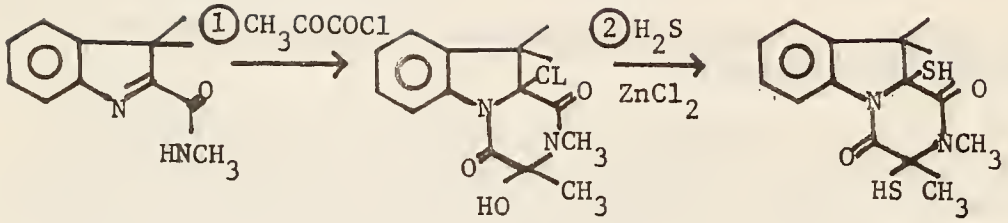
Methods Employed: IR and PMR Spectroscopy, mass spectrometry, thin-layer and column chromatography.

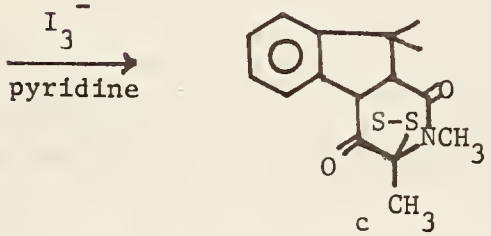
Major Findings: All attempts to synthesize the key intermediate b, in a route to perhydrohistrionicotoxin proposed by E. Gossinger, have failed by reason of failure to form the lithium or magnesium derivatives of a. Exchange reactions, high purity or activated magnesiums or lithium dispersions -- all in a variety of solvents, gave either no reaction or led to dimers derived from a. A possible explanation of the latter is the adsorption of the intermediate radical anion from a onto the metal surface where dimerization is promoted. Such an explanation has been invoked by other investigators to explain the non-reactivity and/or dimerizations of alkyl halides containing polar substituents. The tetrahydropyranyl (THP) protecting group was replaced by the bulkier trimethylsilyl group in an attempt to circumvent this problem but to no avail. At this point this synthetic approach was abandoned. Other routes have been formulated but are not being worked on at this time. In collaboration



a b

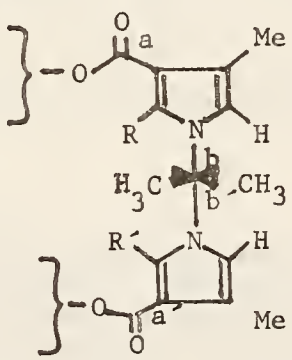
with H.C.J. Ottenheijm, a practical 3-step, one-pot synthesis (83% overall yield) of the gliotoxin analog c was developed. Compound c is a potent inhibitor of reverse transcriptase; its activity is of the same order of magnitude as gliotoxin. The synthesis may be of general applicability in the synthesis of epidithiodioxopiperazines.





c

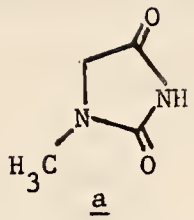
In collaboration with T. Tokuyama, the structure of the three "dimers", A,B and C, obtained by him in the isolation of the frog toxins, batrachotoxin and homobatrachotoxin, and which may be real or artifactural are being studied. Currently, the hypothetical structures shown below are being tested by model studies. Hindered rotations (indicated by study of space-filling models) around bonds a and b could possibly explain the perplexing UV, PMR and mass spectra obtained by T. Tokuyama for these "dimers".



partial "dimer" structures

A: R = R' = Et
B: R = Me R' = Et
C: R = R' = Me

In collaboration with A. N. Radhakrishnan, the structure of a metabolite isolated from the urine of a patient with psoriasis was found to be 1-methyl hydantoin, d. The significance of this compound is not known at this time. It may simply be an artifact of isolation (e.g., from N-carbamyl sarcosine) or it may reflect an abnormal metabolic pathway.



a

Significance to Biomedical Research and the Program of the Institute: Histrionicotoxin analogs should have great utility in probing the function of nerves. Gliotoxin analogs may have a future as antiviral drugs, particularly against oncogenic RNA viruses, which rely on the enzyme reverse transcriptase.

Proposed Course: To devise a new, simple and practical route to perhydrohistionicotoxin.

Publications:

Ottenheijm, H. C. J., Kerkhoff, P. C., Bijen, J. W. H. A. and Spande, T. F.: A three step synthesis of a gliotoxin analog with anti-reverse transcriptase activity. Chem. Comm. 19: 768-769, 1975.

Ottenheijm, H. C. J., Hersheid, J. D. M., Kerkhoff, G. P. C. and Spande, T. F.: An efficient synthesis of a gliotoxin analog with anti-reverse transcriptase activity. J. Org. Chem., submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 19407-02 LC

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Sustained Action Forms of Biologically Active Nucleosides: 2,2,6-Trimethyl-cyclohexane-1-carboxylates

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

PI: James A. Waters	Research Chemist	NIAMDD LC
Other: Gregg Polansky	Stay-in-Schooler	NIAMDD LC

COOPERATING UNITS (if any)
Erik De Clercq, Katholieke Universiteit, Te Leuven, Leuven, Belgium

LAB/BRANCH
Laboratory of Chemistry

SECTION
Metabolites

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	.5	1

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

This project has been discontinued

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19408-03 LC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Analgesics based on the piperidine ring system

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

PI:	J. A. Waters	Research Chemist	LC-NIAMDD
Other:	E. L. May	Scientist Director	LC-NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Metabolites

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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

4-Hydroxy-N-methyl piperidine esters exhibited potency in the codeine-morphine range in the mouse hot-plate assay. Two compounds showed no morphine-like physical dependence capacity in monkeys. Typical narcotic agonist or antagonist activities were absent. Analgesics, which lack the side-effects of morphine, especially physical dependence capacity, and narcotic antagonists are needed for clinical use in relief of pain and in coping with the drug addiction problem.

PHS-6040
(12-75)

Project Description:

Objectives: To design and synthesize compounds from the precursor 4-piperidinol and to assay them for analgesic activity, physical dependence liability, and stereospecific binding to the opiate receptor.

Methods Employed: Numerous synthetic procedures have been used to modify the various positions of the piperidine ring.

Major Findings: Several compounds exhibited potency in the codeine-morphine range in the mouse hot-plate assay. Two piperidinol esters showed no morphine-like physical dependence capacity in monkeys. Typical narcotic agonist or antagonist activities were absent.

Significance to Biomedical Research and the Program of the Institute: Analgesics, which lack the side-effects of morphine, especially physical dependence capacity, and narcotic antagonists are needed for clinical use in relief of pain and in coping with the drug addiction problem.

Proposed Course: Stereoisomers of selected compounds will be prepared for the study of receptor interactions.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 19409-01 LC

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Synthesis of Nucleoside Analogs with Possible Antiviral Properties

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

PI:	Thomas F. Spande	Research Chemist	NIAMDD, LC
Other:	Paul Torrence	Research Chemist	NIAMDD, LC

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemistry

SECTION
Metabolites

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.5	.5

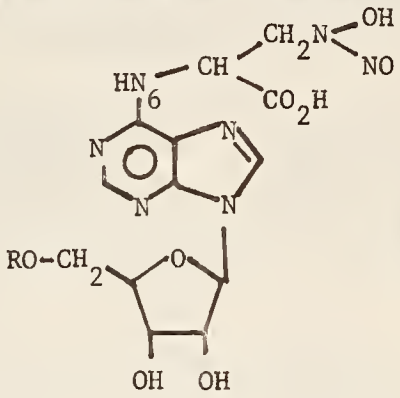
SUMMARY OF WORK (200 words or less - underline keywords)

The synthesis of the alanosine derived nucleoside and necleotide (Ia and Ib) is being pursued in order to determine its effect on adenosine biosynthesis and in order to elucidate the antiviral and antitumor action of alanosine itself.

PHS-6040
(12-75)

Project Description:

Objectives: To synthesize the adenosine nucleoside a and nucleotide b substituted with an alanosine(c) residue in the 6-position as potential inhibitors of adenosine biosynthesis.



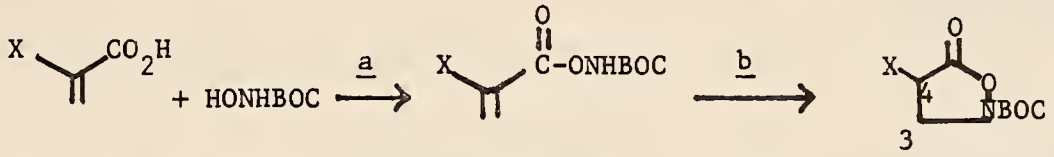
a R = H

b R = PO_3H

Methods Employed: Ir and PMR spectroscopy, mass spectrometry, thin-layer and column chromatography.

Major Findings: Two routes are being explored to prepare appropriately blocked derivatives of c for use with a 6-chloropurine intermediate.

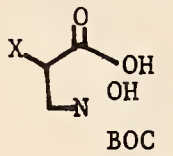
A new synthesis of isoxazolidin-5-ones (5 and 6) has been developed featuring the base-catalyzed cyclization of the acrylic acid esters, 3 and 4, prepared from 1 or 2 and the blocked hydroxylamine derivative. This represents the first synthesis of the saturated isoxazolidin-5-one system, although the 3-4 unsaturated system, found in a number of legume seeds, has been previously synthesized. Intermediates 5 and 6 are easily hydrolyzed with base to the blocked hydroxylamine derivatives 7 and 8. Compound 7 on treatment with ammonia underwent decomposition, necessitating the blocking of the N-OH group with dihydropyran. The blocked derivative 9, on ammonolysis gave 10, although in a slow reaction with a disappointingly low (ca. 10%) yield. All other steps in this sequence were nearly quantitative. It is anticipated that the deblocking of 8 with thiourea will present no difficulty and will permit the direct synthesis of 11 in a more satisfactory, three step synthesis.



1 X = Br
2 X = $NHCOCH_2CL$

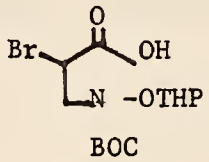
3 X = Br
4 X = $NHCOCH_2CL$

5 X = Br
6 X = $NHCOCH_2CL$



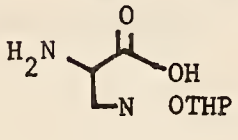
7 X = Br
8 X = $NHCOCH_2Cl$

d on 7



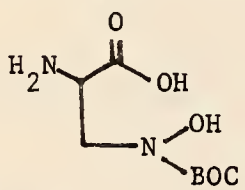
9

e

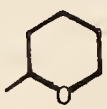


10

e on 7 ✗ | f on 8



BOC = $-CO-O-C(CH_3)_3$

THP = 

a $C_6H_{11}-N=C=N-C_6H_{11}$ - CH_2Cl_2
b Et_3N - CH_2Cl_2(3) or Et $N(iPr)_2$-$^+$BuOH(4)
c Et_3N - H_2O-CH_3CN(5) or $NaHCO_3$-H_2O
d CF_3CO_2H
e NH_4OH
f $(H_2N)C=S$, EtOH-H_2O

Significance to Biomedical Research and the Program of the Institute: The alanosine nucleotide b has been hypothesized as the reaction product from inosine monophosphate and the naturally occurring (Streptomyces alanosinicus) antibacterial, antiviral and antitumor substance, alanosine. This is believed to inhibit adenosyl succinate lyase-the enzyme responsible for converting adenosyl succinate to adenosine monophosphate and furmaric acid, thus blocking the synthesis of this vital RNA component. Synthetic material should be useful in testing this hypothesis and may well have applications as a safe antiviral compound in treating RNA viruses.

Proposed Course: The replacement of Br in 9 with other nitrogen-containing nucleophiles (e.g. N_3^-, phthalimide ion) will be attempted. Improvements in the cyclization of 4 (deacylation is a minor, competing reaction) will be sought. Reaction of the blocked intermediate 10 or 11 with the 6-chloropurine intermediate followed by deblocking and nitrosation (HONO) should provide a. Separation into the L-(natural) and D- diasterioisomers will be attempted by chromatographic and other techniques.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 19600-11 LC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Oxidation Mechanisms in Metabolic Processes

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

PI:

Louis A. Cohen Chief, Sec. on Biochemical Mechanisms LC NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

This project has been temporarily discontinued

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 19601-09 LC

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Chemical Modification and Cleavage of Proteins

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

PI:
Louis A. Cohen Chief, Sec. on Biochemical Mechanisms LC NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Biochemical Mechanisms

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

This project has been temporarily discontinued.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 19602-10 LC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cleavage of Peptide Bonds by Intramolecular Participation

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

PI: Louis A. Cohen Chief, Sec. on Biochemical Mechanisms LC NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

This project has been temporarily discontinued.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 19603-06 LC

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Fluoro Analogs of Enzyme Substrates

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

PI:	Louis A. Cohen	Chief, Sec. on Biochemical Mechanisms	LC NIAMDD
OTHER:	Kenneth L. Kirk	Research Chemist	LC NIAMDD
	John Reepmeyer	Senior Staff Fellow	LC NIAMDD
	Y. Takeuchi	Visiting Associate	LC NIAMDD
	Robert Jerussi	Guest Worker	FDA

COOPERATING UNITS (if any)
Erik DeClercq, Leuven, Belgium
Aviva Lapidot, Rehovot, Israel
Bruce Ames, Berkely, California
Julian Jaffe, Burlington, Vermont

LAB/BRANCH
Laboratory of Chemistry

SECTION
Biochemical Mechanisms

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.5	0.5

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

Our discovery of a photochemical process for synthesis of fluoroaromatics and fluoroheterocycles has led to the synthesis of a large number of ring-fluorinated analogs of metabolically important imidazoles and phenolic amines. 2-Fluorohistidine, but not the 4-fluoro isomer, is incorporated into newly synthesized animal or bacterial protein, in place of histidine. The resulting enzymes may be structurally intact, but some cannot function catalytically because the pK of the imidazole ring has been greatly depressed. The analog serves as a bacteriostatic agent in completely halting the growth of E. coli in two hours. It is also an antiviral agent, blocking viral multiplication in cell culture by generation of false phosphorylases or viral coat protein. The fluoro analog of 5-aminoimidazole 4-carboxamide riboside is also an antiviral agent, blocking the biosynthesis of both DNA and RNA. A variety of fluoroimidazoles, fluorocatecholamines, and fluoroserotonine are being used as tools to study enzyme and receptor mechanisms, as well as neurotransmission.

PHS-6040
(12-75)

Project No. Z01 AM 19603-06 LC

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 had 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 replacement 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 elucidatt product structure. Various chromatographic procedures are used for analysis and purification of mattrials.

Major Findings: The incorporation of 2-fluorohistidine into animal proteins has been further proved by proteoslytic degradation of the protein and identification of the amino acid. 2-Fluorohistidine has been found to inhibit viral multiplication by generating false viral or host cell protein. A lengthy effort to synthesize 2-fluoroimidazole-4-carboxamide has now been completed. 5-Fluoroimidazole-4-carboxamide riboside is converted enzymatically to its triphosphate in whole blood. Studies on the incorporation of tritium into fluoro- and nitroimdazoles have led to the formulation of a new concept, the ALP effect, whereby the lone pair at N-3 blocks exchange at the adjacent C-4 carbon atom. New potential antiviral and antiparasitic agents have synthesized and are being evaluated. A photochemical process has been developed for the first conversion of nitroimidazoles to cyanoimidazoles.

Publications:

Klee, C. B., Kirk, K. L., Cohen, L. A. and McPhie, P.: Histidine Ammonia-Lyase. The Use of 4-Fluorohistidine in Identification of the Rate-determining Step. J. Biol. Chem., 250, 5033-5040, 1975.

De Clercq, E., Luczak, M., Reepmeyer, J. C., Kirk, K. L., and Cohen, L. A.: Fluoroimidazoles as Antiviral Agents and Inhibitors of Polynucleotide Biosynthesis. Life Sciences, 17, 187-194, 1975.

Yeh, H. J. C., Kirk, K. L., Cohen, L. A., and Cohen, J. S.: ^{19}F and ^{1}H Nuclear Magnttic Resonance Studies of Ring-Fluorinated Imidazoles and Histidines. J. Chem. Soc., (Perkin Trans. 2), 928-934, 1975.

Reepmeyer, J. C., Kirk, K. L., and Cohen, L. A.: Synthesis of 5-Fluoro-1-β-D-ribofuranosylimidazole-4-carboxamide, an antiviral agent and inhibitor of polynucleotide biosynthesis. Tetrahedron Letters, 4107-4110, 1975.

Kirk, K. L. and Cohen, L. A.: Biochemistry and Pharmacology of Ring-fluorinated Imidazoles. ACS Symposium Series at Chicago, Illinois in press 1976.

Klein, D. C., Weller, J. L., Parfitt, A., and Kirk, K. L.: 2-Fluoro-L-histidine: An incorporated inhibitor of the adrenergic Stimulation of Pineal N-Acetyltransferase Activity. In Almgren, O., Carlson, A. and Englel, J. (Eds.): Chemical Tools in Catecholamine Research, Vol. II. North-Holland, 1975, pp. 293-300.

Klein, D. C. and Kirk, K. L.: 2-Fluoro-L-histidine: A Histidine Analog which Inhibits Enzyme Induction. ACS Symposium Series at Chicago, Illinois, Biochemistry of the Carbon-Fluorine Bond, in press 1976.

Kirk, K. L.: Photochemistry of Diazonium Salts. IV. Synthesis of Ring-fluorinated Tyramines and Dopamines. J. Org. Chem. in press 1976.

Reepmeyer, J. C., Kirk, K. L. and Cohen, L. A.: 5-Fluoro-1-β-D-ribofuranosyl-imidazole-4-carboxamide. Synthesis of Fluoroimidazole Nucleosides via the Stannic Chloride Coupling Method. In Townsend, L. B. (Ed.): New or Improved Syntheses, Methods, or Techniques in Nucleic Acid Chemistry. Wiley, New York in press 1976.

Klein, D. C., Kirk, K. L., Welles, J. L., Oka, T., Parfitt, A. and Owens, I. S.: 2-Fluorohistidine: An Inhibitor of Enzyme Induction. Molecular Pharmacology in press 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19604-06 LC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

General Principles of Enzyme Catalysis and Simulation

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

PI:	Louis A. Cohen	Chief, Sec. on Biochemical Mechanisms	LC NIAMDD
OTHER:	Paul S. Hillery	Senior Staff Fellow	LC NIAMDD
	Max Blum	Visiting Fellow	LC NIAMDD
	Michael King	Guest Worker	GWU

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	0.5

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

We have advanced the theory that a major portion of enzymatic catalysis is achieved by activation of the substrate during binding. To support the theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen in a single, favorable conformation and by having the interacting functions brought into the closest possible juxtaposition. (stereopopulation control). These compounds undergo intramolecular reactions at rates comparable to those catalyzed by enzymes, sometimes even too fast to measure. It has been possible to generate high energy bonds within these models without energy input, simply using the free energy of formation of water and conformational restriction as the driving forces. In current work, models for sulfhydryl enzymes, oxidative phosphorylation and ion pumps are being developed. Techniques are being developed to elucidate molecular conformation in solution, based on ^{13}C and ^{19}F nmr spectroscopy.

PHS-6040
(12-75)

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^{20}. 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 stereo-population 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 proimity 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: A series of 6-membered thiolactones has been synthesized in order to compare the kinetics of ring closure to oxygen and the higher energy sulfur lactones. Despite the chemical similarity between the two hetero atoms, serious obstacles were encount red in the synthesis and kinetic analysis in the sulfur series: rearrangement of aromatic ring substituents, air oxidation, and an extremely powerful chelating ability for trace metals. These obstacles have been largely surmounted, and kinetic studies are in progress. The same substrates will be used to study sulfhydryl cleavage of amides, as models for enzymes such as papain.

Initial exploration has been made in the use of ^{19}F nmr as a probe of solution conformation. The temperature dependence observed for the collapse of fluorine doublets indicates rotational barriers considerable higher than predicted for the trialkyl lock.

Kinetic studies with five-membered phenolic lactones have been completed, and show that rate inhancement is due entirely to elevation of ground state free energy, and not to product structure. This concept has been further validated by mass spectral measure of ^{18}O exchange rates into lactones of various geometrics.

Significance to Bio-medical Research and the Program of the Institute: Despite the vast amount of research invested in the elucidation of the pathways of specific enzyme-catalyzed reactions, justification of the rates of these reactions, in comparison with their nonenzymatic counterparts, are still in a primitive state. A sound understanding of the means by which enzymes

operate so effectively would lead not only to a better appreciation of biochemical processes in general, but also to a more rational approach to drug design.

Proposed Course: Utilizing the principles of stereopopulation control already developed, additional models will be studied to produce even faster reaction rates than those already observed. The principle will also be extended to other types of reactions, such as hydride transfer and addition/elimination. The control principle will also be used to design conformationally restricted analogs of various drugs with a view to enhancement of activity and reduction of side-effects.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19801-31 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Service Function and Instrumentation

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

PI:	D. F. Johnson	Section Chief	LC-NIAMDD
Other:	H. Y. C. Yeh	Senior Staff Fellow	LC-NIAMDD
	W. R. Landis	Chemist	LC-NIAMDD
	P. Parisius	Chemist	LC-NIAMDD
	B. Baer	Chemist	LC-NIAMDD
	A. Wong	Chemist	LC-NIAMDD
	N. Whittaker	Chemist	LC-NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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

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 government agencies. Approximately 30 of the more common elements and five functional groups are 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 when requested. Instrumental analyses include: GC/MS spectrometry, gas-liquid chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, atomic absorption spectrophotometry, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request.

PHS-6040
(12-75)

Project Description: 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 government agencies. Approximately 30 of the more common elements and five functional groups are determined on a quantitative basis, using ultramicro, micro, and semi-micro techniques as required. The materials analyzed include organic and inorganic research samples, commerical preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents when requested. Instrumental analyses include: GC/MS spectrometry, gas-liquid chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, atomic absorption spectrometry, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request.

During the past year the total number of analyses performed in the microanalytical laboratory was 10,914, roughly a 19% decrease in the total from the the last reporting period. This represents a normal change in as much as last years report reflected a 20% increase over the previous reporting period. Review of the special types of analyses are shown below;

Analyses Run

	1974-75	1975-76	% Change
Carbon	3449	2857	-17.2
Hydrogen	3449	2857	-17.2
Nitrogen	5105	4105	-19.6
Phosphorous	136	191	40.4
Sulfur	99	95	- 4.0
Halogens	263	375	42.6
Metals	434	133	-69.4
Weight Loss	222	64	-71.2
Rotations	23	63	173.9
Miscellaneous	247	174	-29.6
	13,427	10,914	-18.7

The distribution of service rendered is LC (33%); NIH, not including LC (64%); and outside agencies (3%). This distribution is comparable to the previous reporting period.

Very little service work was performed on the Perkin-Elmer 421 infrared spectrometer. The bulk of this type of analysis is done by individual investigators on low resolution infracords, and our instrument is only used for special studies. It is retained however because it is the only such instrument available to LC.

The NMR service includes both the Varian A60 and HA-100. The major share of service work is done on the former. Over 200 spectra were run on the A60 by Mr. Benjamin Miller under the guidance of Mr. Noel Whittaker. Additional spectra were run by individual investigators. Mr. Miller also maintains the documentation and stocking of organic chemical stores for the laboratory. As an additional service function in NMR. Dr. Herman Yeh has run 500 spectra on the Varian HA-100 and 50 on a Varian HR-220 (located in a different section). These specialized spectra were, in addition to Dr. Yeh's research program, reported elsewhere.

More than 1300 samples were submitted for mass spectral analysis on the Hitachi Perkin-Elmer RMU-6E. Each analysis involved many spectral determinations. Standard qualitative spectra were routinely completed along with other analytical requests of the research investigator: isotope quantitative mass-ratio measurement; on-scale spectra recorded either/or the visicorder and the pen recorder, and sample mixtures requiring fractional volatility-changing rate separations, introduced through the solids probe.

In accordance with our instrument's current limited ability to obtain high-resolution mass measurement via the existing manual "peak matching" instrumentation, the laboratory is considering the purchase of a compatible data system. This system would provide automatic, computerized exact-mass determination, plus other intrinsic features capable from our mass spectrometer, but now not usable in its existing mode.

The number of samples analyzed were:

NIAMDD - Laboratory of Chemistry:	1116
N. I. H., other than NIAMDD-LC:	189
Outside the N. I. H.:	46
Total Samples Analyzed	1351

Mr. Noel Whittaker is the principal operator of the Finnigan GC/MS system. This system is a recent addition to the instrumentation group and is used very heavily by the investigators. Approximately 575 samples were run by solid probe in the CI mode. An additional large number of samples were run using GC/MS. These include separation and indentification of amino-acid metabolites from liver homogenates, alkaloid mixtures from frog skin extracts, mixtures of metabolites from bromoanisoles, and mixtures of benzopyrenes, to mention but a few. In addition to the service work for investigators in LC. Mr. Whittaker has been involved with a number of collaborative studies with outside investigators when time permits. Equipment was purchased and fabricated here at NIH to allow for EI spectra to be done on the Finnegan with the greater sensitivity in a low pressure source. Work to make this system operational continues.

The Section Chief continues to serve as Project Officer on an NIAMDD contract with the Medical Research Council of England. This contract provides reference steroid compounds to qualified research workers in the United States.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19802-03 LC

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Characterization of Steroid Transforming Enzymes in Tetrahymena Pyriformis

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

PI:	Nancy S. Lamontagne	Research Chemist	LC-NIAMDD
Other:	David F. Johnson	Section Chief	LC-NIAMDD

COOPERATING UNITS (if any)

Chester E. Holmlund, Department of Chemistry, University of Maryland, College Park, Maryland

LAB/BRANCH
Laboratory of Chemistry

SECTION
Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0	1.0

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

The recent isolation of 4-androstene-3β,17β-diol as a transformation product from incubation T. pyriformis with testosterone in this laboratory indicates that this protozoan has a dehydrogenase capable of reducing the 3-ketone of a steroid to a 3β-hydroxyl. Our objective is to purify and characterize the nature of this enzyme. The identification of several other transformation products is also being pursued in the hope of establishing that T. pyriformis has an isomerase that can transform a Δ^4-steroid to a Δ^5-steroid.

PHS-6040
(12-75)

Project Description: Since Tetrahymena pyriformis appears to have enzyme systems comparable to those in higher animals, investigation of steroid transformations (and the enzymes involved) in this model system could help in the understanding of steroid metabolism in mammalian systems.

The isolation of 4-androstene-3β,17β-diol as a transformation product from the incubation of T. pyriformis with testosterone indicates that this protozoan does have a dehydrogenase capable of reducing the 3-ketone of a steroid to a 3β-hydroxyl. Our objective is to demonstrate the presence of a β-hydroxysteroid dehydrogenase (β-HSDH) in T. pyriformis, and to purify and characterize the nature of this enzyme.

The optiomal growing conditions (e.g. culture age, amount of aeration) for T. pyriformis, and the optimal storage conditions (e. g. stabilizing reagents, temperature) of the crude supernatant from the harvested cell homogenate have been determined. It has also been determined that the β-HSDH activity is not inducible by steroids or related compounds. Although the amount of constitutive β-HSDH activity in T. pyriformis is small, the development of a quick and reproducible spectrophotometric assay for the enzyme, and its relative stability will make the subsequent purification feasible. A variety of standard enzyme purification techniques are being used in the hope of isolating a sufficient amount of β-HSDH for equilibrium and kinetic studies.

The identification of several other transformation products from the incubation of T. pyriformis with progesterone is being pursued in the hope of establishing that T. pyriformis can isomerize a Δ^4-steroid to a Δ^5-steroid, as well as reduce a carbonyl group to a hydroxyl.

Publications: Lamontagne, Nancy S., Johnson, David F., and Holmlund, Chester E.: The Transformation of Testosterone by Tetrahymena pyriformis. J. of Steroid Biochem. 7: #3, 177-183, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19803-03 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Applications of NMR in Biochemical and Biological Systems

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

PI:	Herman J. C. Yeh	Senior Staff Fellow	LC-NIAMDD
Other:	D. F. Johnson	Section Chief	LC-NIAMDD
	L. A. Cohen	Section Chief	LC-NIAMDD
	K. L. Kirk	Research Chemist	LC-NIAMDD
	J. S. Cohen	Research Chemist	RRB NICHD
	D. M. Jerina	Research Chemist	LC-NIAMDD
	H. Yagi	Visiting Associate	LC-NIAMDD
	A. E. Jacobson	Research Chemist	LC-NIAMDD
	O. Hernandez	Visiting Fellow	LC-NIAMDD
	W. A. Klee	Research Biochemist	LGCB NIMH
	R. S. Wilson	Research Chemist	LC-NIAMDD
	G. M. Holder	Research Chemist	LC-NIAMDD
	P. M. Dansette	Research Chemist	LC-NIAMDD

COOPERATING UNITS (if any)

K. N. Chen, R. M. Moriarty, B. G. Deboer, and M. R. Churchill (U. of Illinois at Chicago Circle); D. T. Gibson and V. Mahadevan (U. of Texas at Austin); H. A. Berman and T. R. Stengle (U. of Massachusetts).

LAB/BRANCH

Laboratory of Chemistry

SECTION

Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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

The purpose of this project is to develop nuclear magnetic resonance methods for elucidating molecular structure and for studying the interactions within and between molecules, especially those of biological interests.

PHS-6040
(12-75)

Project Description: Basic information on the structure and molecular interactions of biologically important materials is of significance to biochemical and biological research throughout NIH. One of the most promising approaches in obtaining such information is through the use of nuclear magnetic resonance. The purpose of this project is to develop nuclear magnetic resonance methods for studying molecular structures and molecular interactions in biochemical and biological systems.

Various NMR methods (e.g. selective decoupling experiment, lanthanide shift experiment, etc.) have been used successfully to assign the structures of compounds such as the dihydrodiols (derived from bacterial oxidation of the carcinogens benzo[a]pyrene and benzo[a]anthracene), the isomeric hydroxybenzo-[a]pyrenes, the halomorphides (narcotic drugs) and the ferrelactones (reaction products of iron carbonyl with diastereomeric 2,3-oxidohex-4-enes).

[^{31}P]-^{1}H NMDR technique coupled with pD titration has been used to study the interaction between phosphate containing molecules, 4-deoxypyridoxine phosphate and monomethylphosphate, and a number of structurally related biogenic amines. The ^{31}P NMR results show a relation between amine structure and the strength of binding to the phosphate groups of the model compounds. A remarkable correlation between these NMR results and pharmacological binding study is found which tends to support the hypothesis that the phosphate group may in some way be related to the amine binding sites within the storage vesicles of adrenergic nerve terminals.

^{19}F and ^{1}H NMR have been used to investigate the effect of local charge on ^{19}F and ^{1}H chemical shifts in 2-fluoro and 4-fluoro histidines, which from some early studies have indicated their biological significance in analogy to histidine. A large shift in ^{19}F over ^{1}H NMR signals is observed in both compounds as the side chain groups of these molecules being titrated, illustrative of the high sensitivity of ^{19}F signals to distant field effects. In complex systems, like proteins, the use of ^{19}F as an NMR reporter also offer an advantage over that of ^{1}H, since overlap or concealment by neighboring signals can be eliminated. Presnent study suggests that the use of ^{19}F NMR for the study of protein systems is encouraging.

[^{15}N]-^{1}H NMDR has been used to obtain both proton and nitrogen-15 spectra of a series of ^{15}N labeled 2-acylpyrroles. ^{15}N chemical shifts for these compounds are reported for the first time. No correlation between the nitrogen chemical shift and any Hammett substiutent constant could be found. No variation in J (^{15}NH) was observed for each compound with changes in solvent, temperature, or concentration ruling out any observable tautomeric equilibria for these systems. An increase in J (^{15}NH) with the addition of electron withdrawing groups indicates increasing polarization of the NH bond and acidity of these molecules.

Investigations on the heme-ligand interactions of metalloporphyrins and their hemoproteins is in progress. NMR study on the chemical structure and biological function of hemerythrin, an oxygen-transport pigment found in erythrocytes of the coelomic fluid of certain invertebrates, is being planned.

Publications:

Gibson, D. T., Mahadegan, V., Jerina, D. M., Yagi, H. and Yeh, H. J. C.: Oxidation of the carcinogens benzo[a]pyrene and benzo[a]anthracene to dihydordiols by bacteria. Science 189: 295-297, 1975.

Yeh, H. J. C., Kirk, K. L., Cohen, L. A. and Cohen, J. S.: ^{19}F and ^{1}H nuclear magnetic resonance studies of ring-fluorinated imidazole and histidine. J. Chem. Soc. (Perkin II) 928-934, 1975.

Chen, K. N., Moriarity, R. M., DeBoer, B. G., Churchil, M. R. and Yeh, H. J. C.: Stereochemical course of the reaction of iron carbonyl with diastereomeric vinyloxiranes. J. Am. Chem. Soc. 5602-5603, 1975.

Yeh, H. J. C.,Wilson, R. S., Klee, W. A. and Jacobson, A. E.: α- and β- Halomorphides: stereochemistry, analgesic potency, toxicity and their interaction with narcotic receptors in vitro. J. Pharmaceut. Sci. in press, 1976.

Berman, H. A., Yeh, H. J. C. and Stengle, T. R.: Contact ion assoication of perchlorate ion. A ^{35}CL NMR Study. II. Solutions in mixed solvents. J. Phys. Chem. 79: 2551-2555, 1975.

Yagi, H., Holder, G. M., Dansette, P. M., Hernandez, O., Yeh, H. J. C., LeMahieu, R. A. and Jerina, D. M.: Synthesis and spectral properties of the isomeric hydroxy-benzo[a]pyrenes. J. Org. Chem. 41: 977-985, 1976.

King, M. M., Yeh, H. J. C. and Dudeck, G. O.: Nitrogen nmr spectroscopy-application to some substituted pyrroles., Org. Mag. Resonance in press, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19804-03 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Development of Chemical Methods and Compounds for the Study of Biology and Medical Problems

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

PI: Calvin M. Foltz Research Chemist LC-NIAMDD
Other: None

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Chemistry

SECTION

Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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

The primary goal of this work is to contribute to the investigation and solution of basic biological and medical problems. This is to be done by the development of chemical methods and reagents for the study of the primary, secondary, tertiary, and quaternary structures of biomacromolecules, for the study of the structure and properties of organelles and cell components, and for the selective modification of biomacromolecules; by the study of intra- and intermolecular interactions; and by the study, development, and application of organic and photochemical reactions. Areas of special interest at present are photocyclization, photorearrangements, and sellenium chemistry.

PHS-6040
(12-75)

Project Description: The primary goal of this work is to make contributions to the investigation and solution of basic biological and medical problems by the application of chemistry and physical methods.

One of the current goals of this project is the completion of work in progress on the extension of the photocyclization observed with N-chloroacetyltryptoplain {Yonemitsu, Cerutti and Witkop (1966)} to tryptamine and derivatives and homologs of tryptamine. The tricyclic lactams produced are of chemical and potential biological and medical interest. The reaction was extended to non-heterocyclic systems with the photolysis of N-chloroacetyl-2-(α-naphthyl)ethylamine {Foltz, J. Org. Chem., 36, 24 (1971)} and this work continued in the above and other systems will yield compounds of chemical, biological and medical interest and possibly methods for the modification of biological molecules and structures. Additional work is also in progress on the study of the scope, mechanism and applications of the interesting rearrangement observed on photlysis of 3-(3'-chloro-2'-oxopropyl)-indolin-2-one {Foltz and Kondo, Tetrahedron Letters, 3163 (1970)}.

In 1957 the discovery of the fact that the dietary agent Factor 3, which prevented dietary liver necrosis in the rat, supplied trace amount of selenium was reported {Schwarz and Foltz, J. Am. Chem., 79, 3292 (1957)}. This report has stimulated a large and growing body of work on the biological and nutritional roles of selenium.

Recently two selenoenzymes and a selenoprotein have been reproted {reviewed by Statdtman, Science, 183, 915 (1974)}. Developments in this area are being followed closely and work in the field is anticipated as opportunities present themselves for making contributions to knowledge of the biological role of selenium.

Publications: None

NATIONAL SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19805-01 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Gonadal Steroid-Stimulated Formation of Cyclic AMP in Incubated Rat Hypothal

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

PI:	B. A. Weissman	Visiting Fellow	LC-NIAMDD
Other:	D. F. Johnson	Section Chief	LC-NIAMDD
	P. Skolnick	Staff Fellow	LC-NIAMDD
	J. W. Daly	Section Chief	LC-NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

Work has been discontinued.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19806-01 LC

PERIOD COVERED

September 29, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Nature of Steroid-Receptor Interaction

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

PI:	S. S. Simons, Jr.	Staff Fellow	LC-NIAMDD
Other:	D. F. Johnson	Section Chief	LC-NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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

The purpose of this project is to define the preliminary steps in steroid hormone action. In particular, a line of glucocorticord-responsive rat hepatoma tissue culture cells will be used to look at: 1) steroid-receptor binding site interactions; 2) effects of steroid binding on receptor conformation; 3) the nature of "activation" of receptor-steroid complexes, and 4) the nuclear binding of activated receptor receptor-steroid complexes.

PHS-6040
(12-75)

Project Description: A line of glucocorticoid responsive rat hepatoma tissue culture (HTC) cells has been used extensively to study specifically the mechanism of action of steroid hormones and, more generally, the control of gene transcription in eukaryotes. Previously we found that activated receptor steroid complexes bind to HTC cell nuclei, chromatin and DNA in a process that is inhibited by an interaction of unknown cytoplasmic components with the activated complexes (Simons, Martinez, Garrea, Baxter and Tomkins, (1976) J. Biol. Chem. 251: 334-343, and Simons, submitted for publication). The affinity of activated complex binding to acceptors appears to be influenced by chromosomal proteins and nucleotide sequence. Furthermore, we previously determined the probable existence of two forms of the activated complex.

We have undertaken an investigation of some of the questions suggested by these recent results. In particular the nature of the cytoplasmic inhibitor(s) and their effect, if any, on receptor-steroid interactions and transformations will be studied. A detailed understanding of these phenomena is particularly desirable since therapeutically valuable, steroid-specific differences are most likely expressed at this level in steroid hormone action. Finally the nature of the nuclear acceptor site for activated complexes will be examined further.

While conventional methods will be used in these investigations, the use of novelly derivatized glucocorticoids will also be employed. This latter method has the potential of obtaining otherwise difficultly accessible data and has been the object of most of our efforts so far.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 20000-09 LC

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

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

PI:	C. R. Creveling	Research Chemist	LC NIAMDD
OTHER:	J. W. Daly	Chief, Sec. on Pharmaco.	LC NIAMDD
	A. Rotman	Visiting Fellow	LC NIAMDD

COOPERATING UNITS (if any) B. Hartman, Washington U., St. Louis,MO; C. Isersky, NIAMDD-CI; L. Tice, NIAMDD-LEP, K. Inoue, NIAMDD-LEP; B. Highman, Nat. Center Toxicol. Res., Jefferson, AK: P. Siekevitz, Rockefellar U.,NY; R. Cohen, Rockefeller U.; B. Nikodijevic, U. Skopje, Yugoslavia; D. Klein, NICHHD-LBS; B. Bernard, U. Conn. P. Ghosh, Guest worker, Howard U., Wash. D. C.

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Pharmacodynamics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	1.5	2.5

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

The purpose of this project is to study the chemistry and biochemistry of biogenic amines, their amino acid precursors and transformation products. The areas of specific interest are: 1) the mechanism of the neurotoxicity of 6-hydroxydopamine, 5,7-dihydroxytryptamine and related compounds; 2) the role of adrenergic innervation in the release of calcitonin from the C-cell of the thyroid; 3) the histopathology of nonspecific toxic effects of dihydroxytryptamines on cardiac muscle; 4) the effect of fluorine derivatives of biogenic amines on transport mechanisms in vivo, in atrial preparations and in platelet preparations; 5) the localization of both soluble and membrane bound catechol-O-methyl transferase through the use of antibodies; and 6) the incorporation of 2-fluorohistidine into protein in vivo.

PHS-6040
(12-75)

Project Description:

Objectives: To Study the basic chemistry and biochemistry of the catecholamines, phenolic amines, indolealkylamines, imidazolealkylamines and their precursors and metabolic transformation products. To develop new techniques for the investigation of membrane transport systems and receptor binding phenomena as related to these amines and their interaction with various drugs. To study the mechanism of action of specific neurodegenerative agents structurally related to these amines; to investigate the specific sites of the cytoxic action of these compounds and describe the subcellular morphological changes which accompany this action. To study the histopathology of the non-neuron specific effects of thse cytotoxic amine derivatives. To isolate and purify enzymes operative in the biosynthesis and metabolism of biogenic amines. To determine the physical properties of these enzymes. To develop assays and to apply these enzymatic assays to determine specific biogenic amines levels in tissue and to study the distribution and variation of enzyme levels. To prepare specific antibodies for these enzymes and apply the antibodies to the development of radioimmune assays for the assay the localization of these enzymes. To investigate the distribution of these enzymes at both the cellular and subcellular level thru the application of immunoperoxidase and ferritin labelling techniques and by immuno fluorescent techniques.

Methods Employed: Drugs and enzymes isotopically labeled with tritium, deuterium, iodine-125 or carbon-14 have been prepared to study the metabolic fate of endogenous amines and amino acids and the mechanism of membrane transport and release of these compounds. Standard methods of organic synthesis have been used to prepare these compounds. Standard methods of protein purification have been used to purify these various enzymes as well as the development of specific affinity labeling techniques and immunoabsorption techniques. Standard methods of histopathology employing light and electromicroscopy have been used to examine cytotoxic effects of neurotoxic amines. Standard immunodiffusion, electrophoresis and immunization techniques have been employed in the development of antibodies for these enzymes.

Major Findings: The effects of 6-hydroxydopamine, 5,7-dihydroxytryptamine and several related compounds were examined with regard to the mechanism of neurocytotoxicity in model systems. An oxygen-dependent binding and subsequent cross-linking of model proteins was demonstrated. This binding and cross-linking phenomena occurred primarily through free sulfhydryl groups on proteins. Attempts were made to relate this model for cytotoxic action to events in neurobastoma cultures, in isolated mouse atrial preparations and in intact mice. 6-Hydroxydopamine is bound irreversibly to atria both *in vitro* and *in vivo* and results in a deficit in the active transport system for the normal transmitter, norepinephrine. Similar results were obtained with 5,6-dihydroxytryptamine *in vitro*. While the present results indicate a relationship between the irreversible binding of neurotoxic amines and the cytotoxic sequelae, no direct evidence for amine-induced protein-polymerization in isolated atria or *in vivo* has been obtained. (Rotman)

A study of the effect of 6-hydroxydopamine and related compounds on the morphology and response to Ca^{++} of the calcitonin-containing cells of mouse thyroid have demonstrated a relationship between sympathetic innervation of the thyroid and the release and regranulation in the calcitonin-containing cell. However, no evidence was obtained for a cytotoxic effect of 6-hydroxydopamine in either thyroid or calcitonin cells. A neurotoxic degeneration of sympathetic neurons in the thyroid followed administration of either 6-hydroxydopamine, 6-hydroxydopamine and 5,7-dihydroxytryptamine. (Creveling)

The cytotoxicity of 5,7-dihydroxytryptamine *in vivo* is blocked by prior inhibition of monoamine oxidase. The possibility that high concentrations of norepinephrine in monoamine oxidase-inhibited animals might block the cytoxicity of 5,7-dihydroxytryptamine through provision of an "electron-sink" which would prevent the accumulation of toxic levels of 'superoxide ion' or hydroxyl ion was investigated. However, animals with lowered endogenous levels of norepinephrine, resulting from either depletion by reserpine or inhibition of synthesis by α-methyl-*p*-tyrosine or both, showed the same sensitivity to 5,7-dihydroxytryptamine and the same protection by monoamine oxidase inhibition. This result supports the binding and cross-linking mechanism for the mechanism of action of cytotoxic amines and suggests that the formation of superoxide radical through oxidation by the amine is not the primary mechanism of cytotoxicity. (Creveling)

A study of the histopathological effect of the dihydroxytryptamine demonstrated that, 1) 6,7-dihydroxytryptamine was acutely toxic (ED_{50}= 30 mg/kg) in mice and that the toxicity was related to ischemic responses in the vaculature leading to necrotic lesions of vessel walls; 2) administration of 5,6-dihydroxytryptamine resulted in a dose and time dependent development of subepicardial lesions of the myocardium which was preceded by a fatty infiltration of myocardial fibers; 3) the remaining dihydroxytryptamines including 5,7-dihydroxytryptamine fail to produce any cardiovascular toxicity and 4) that there is no apparent relationship between the specific neurocytotoxicity and the effects on either the vasculature or the myocardium. (Creveling)

Routine studies on the inhibition of norepinephrine uptake and the release of norepinephrine both *in vivo* and in isolated atrial slices are being continued to find additional compounds which may be useful as investigational tools. The kinetic parameters of the uptake mechanism are currently under study as a model for investigating the mechanism responsible for the transport of amine through the neuronal membrane. An examination of the transport and storage of the fluorotyramines, dopamines and indoleamines in atrial preparations are being examined. The isolated atrial system *in vitro* is being used as a model to measure the release of amines stored intraneuronally. The possible involvement of S-adenosyl-L-methionine-dependent carboxymethyltransferase in the release of amines is being examined through the use of product inhibition of the transferase. (Creveling)

Evidence for the cellular localization of both soluble and "membrane" bound catechol-O-methyltransferase has been obtained through the use of specific antibodies to this enzyme in conjunction with the "PAP" technique and with ferritin-labeled antibody. The enzyme has been tentatively localized in the 'microsomal' or reticuloendothial processes in liver cells. Present studies are directed at a survey of other peripheral sites including the kidney, heart, vasculature and superior cervical ganglia. Localization studies in the brain are progressing more slowly. The antigenicity of the endogenous enzyme in brain is subject to variation with various biochemical treatments and thus may be located in forms which shield the enzyme from antibody attack. (Creveling)

A study of the incorporation of 2-fluorohistidine into mouse protein, in vivo, are being continued. The histopathology of 2-fluorohistidine is being examined in adult and young mice. Efforts have been directed towards a demonstration of the incorporation of 2-fluorohistidine in place of histidine in the intact mouse, in organ cultures of rat pineal glands and in globin synthesized de novo with a rabbit reticulocyte system. (Kirk, Creveling)

Significance to Biomedical Research and the Program of the Institute: The biosynthesis, uptake and metabolism of biogenic amines are of fundamental importance to the fields of neurochemistry and pharmacology and have a direct bearing on the understanding of the functioning of the cardiovascular system and the central biogenic amines-containing neurons. The nature of the localization of catecho-O-methyltransferase both soluble and 'membrane' bound and the variation of this enzyme among species and under vaious pathologic conditions may provide insight into the etiology of essential hypertension. An understanding of the mechanism and mechanisms of action of cytotoxic amines will not only provide a rational for the design of additional specific cytotoxic agents but may provide guidelines for the application of such agents towards other cell types including neoplastic cells.

Proposed Course of Project: The mechanism of cytotoxicity of various neurotoxic amines will be studied in various model systems and attempts will be made to define the mechanism operative in vivo. Studies on the nature of the active transport system for amines in the plasma membrane of noradrenergic neurons will be continued with special emphasis upon the involvement of cytoxic amines. Studies on the localization and structural characteristics of "membrane" bound catechol-O-methyltransferase will be continued with special emphasis on the possible role of this enzyme as a component of the postsynaptic membrane in catecholaminergic neurons of the brain. The interaction of fluorine derivatives of biogenic amines with both uptake and storage mechanisms as well as noradrenergic and serotinergic receptors will be continued. The effects of both 2-fluorohistidine incorporation into protein in place of the normal precursor and the metabolism through decarboxylation to 2-fluorohistamine will be continued.

Publications:

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Tice, L. W. and Creveling, C. R.: Electron microscopic identification of adrenergic nerve endings on thyroid epithelial cells. Endocrinol. 97: 1123-1129, 1975.

Rotman, A., Daly, J. W., Creveling, C. R. and Breakefield, O.: Uptake and binding of dopamine and 6-hydroxydopamine in murine neuroblastoma and fibroblast cells. Biochem. Pharmacol. 25: 383-388, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 20001-06 LC

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Role of Cyclic Nucleotides in the Nervous System

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

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	4.5	1.5

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

The role of cyclic nucleotide-generating systems to the functional operation of the central nervous system is being investigated from a number of standpoints: i) Alterations in the levels or properties of brain adenylate cyclases, phosphodiesterases and protein kinases as a result of pretreatments of animals with drugs, hormones, or environmental manipulation. ii) The nature and homeostatic control of cyclic AMP and cyclic GMP-generating systems in brain slices with emphasis on the types of neurotransmitters and antagonists which interact with the systems. iii) Correlation of effects of drugs on the function of cyclic AMP and cyclic GMP-systems in brain slices and cell-free preparations with in vivo effects of the drugs on behavior and vegetative function. iv) Strain and species differences in the magnitude and character of responses of cyclic AMP-generating systems in brain slices to catecholamines, serotonin, histamine, adenosine, prostaglandin and depolarization and the sensitivity of such animals to drugs affecting these neurotransmitter substances. v) The nature and control of the interactions of ions such as calcium, magnesium, manganese and zinc with the cyclic nucleotide-generating systems of brain.

Project Description: Project No.Z01 AM 20001-06 LC

Objectives: To determine the factors that govern the formation and metabolism of cyclic AMP and cyclic GMP in the central nervous system. To investigate the possible role of cyclic AMP in regulating biochemical reactions and membrane properties in brain tissue. To investigate the interrelationship between central neurotransmitter function and cyclic nucleotides. To investigate the role of adaptive changes in cyclic nucleotide-generating systems in drug-tolerance and dependence.

Methods Employed: Surgical techniques, environmental manipulation, drug-regimens have been used to alter central function. Formation and metabolism of cyclic nucleotides in biological preparations have been studied with isotopically labeled compounds.

Major Findings: The nature of noradrenergic receptors controling cyclic AMP-formation in brain tissue has been further characterized. Clonidine, normally considered to be an α-agonist, has been found to interact with cyclic AMP-generating systems at presynaptic and postsynaptic loci in rat cerebral cortical slices. At the postsynaptic loci it blocks the α-adrenergic component of norepinephrine-elicited accumulation of cyclic AMP, and as an α-agonist greatly potentiates the accumulation of cycic AMP-elicited by isoproterenol. Clonidine, in addition, reduces basal levels of cyclic AMP in phenoxybenzamine-treated slices apparently by interacting as an inhibitory agonist at presynaptic α-receptors which control release of norepinephrine. (Skolnick)

Two classes of β-noradrenergic receptors controlling cyclic AMP-generating systems have been detected in cerebellar slices from rats. One β-receptor apparently present in Purkinje neurons is inhibited by β-antagonists and by neuroleptic phenothiazines such as fluphenazine. The other class of β-receptors is apparently associated with neurons of the granule layer and is not inhibited by neuroleptics. This receptor type is absent in cerebellar slices from rats in which the granule neurons were destroyed by neonatal x-irradiation. (Skolnick)

The levorotary isomer of alprenolol, normally considered a very specific β-antagonist and a useful tool for binding studies with β-receptors, is found to inhibit both the α- and β-adrenergic components of (nor)epinephrine-elicited accumulation of cyclic AMP in rat cerebral cortical slices. (Skolnick)

The α-component of norepinephrine-elicited accumulations of cyclic AMP in rat brain slices has been shown to be completely dependent on extracellular calcium ions. In contrast, the β-component of norepinephrine-elicited responses of cyclic AMP-generating systems in brain slices is only partially reduced by the absence of extracellular calcium ions. (Schwabe)

Adenosine deaminase reduces basal levels of cyclic AMP within brain slices, presumably by enzymatic inactivation of "released" adenosine within the slice. Adenosine-antagonists such as theophylline also reduce basal levels presumably by antagonizing adenosine-elicited activation of cyclic AMP-generating systems. In the presence of adenosine deaminase, responses

of cyclic AMP-generating systems to amines are markedly reduced, while responses to N^6-phenylisopropyladenosine, a nonsubstrate for the deaminase, are unaffected or somewhat reduced depending on the species. Thus, endogenous adenosine appears important to responsiveness of cyclic AMP-systems in brain slices. This is particularly true in the absence of extracellular calcium. Under such conditions adenosine deaminase completely prevents any response to biogenic amines. (Schwabe)

Norepinephrine, adenosine and a depolarizing agent, veratridine, elicit accumulations of cyclic AMP and cyclic GMP in rodent brain slices. In addition, calcium ions added to cerebellar slices preincubated in calcium-free medium elicits a rapid 2 to 7-fold accumulation of cyclic GMP, while having little or no effect on basal levels of cyclic AMP. The magnitude of the calcium-elicited accumulation of cyclic GMP is dependent on the species used as source of the cerebellar slices and is greatest with guinea pig. Neurotransmitter release did not appear to be involved in either calcium or veratridine-elicited accumulations of cyclic GMP. Veratridine-elicited accumulation of cyclic AMP appears strongly dependent on depolarization-elicited release of adenosine. The calcium-elicited accumulation of cyclic GMP in guinea pig cerebellar slices is not effectively inhibited by many so-called calcium antagonists or potentiated by calcium ionophores. Of the many compounds tested, promethazine is the most potent antagonist of calcium-elicited accumulations of cyclic GMP, with diphenhydramine, brompheniramine, chlorpromazine and imipramine less active. These compounds were only relatively weak inhibitors of cerebellar guanylate cyclases. (Ohga)

Disruption or reduction of noradrenergic transmission in the central nervous system results in rat in a marked compensatory hyperresponsiveness of norepinephrine-sensitive cyclic AMP-generating systems in rat brain slices. Surprisingly depletion of norepinephrine with reserpine, lesions of ascending noradrenergic neurons of the medial forebrain bundle or destruction of noradrenergic terminas with 6-hydroxydopamine has no effect on responses of cyclic AMP-systems to norepinephrine in guinea pig brain, as measured in cerebral cortical slices. The normally highly responsive norepinephrine-sensitive cyclic AMP-generating systems in cortical slices of certain rat strains also do not show adaptive increases after 6-hydroxydopamine. Thus, it appears likely that in certain species and strains of animals a genetically low functional noradrenergic input has already resulted in maximally responsive cyclic AMP-systems. (Creveling, Dismukes, Ghosh, Skolnick)

The responsiveness of cyclic AMP-generating systems has been compared in brain slices of rats reared in enriched or impoverished environments. Alterations in responsiveness might then provide indications of heightened or reduced functional activity of certain neurotransmitter systems as a result of environmental input. Responses to histamine appeared to be higher in "enriched" rats, while responses to prostaglandin were higher in "impoverished" rats. (Dismukes)

Accumulations of cyclic AMP elicited by norepinephrine and adenosine in cerebral slices from eight strains of rat have been ascertained. No clear correlations among this data and spontaneous open field activity or active avoidence learning were apparent either between strains or between individuals of an outbred strain. This contrasts to previous data indicating a negative correlation between norepinephrine-elicited accumulations of cyclic AMP in cerebral slices of four rats strains and the spontaneous behavioral activity. (Dismukes, Stalvey)

A new class of phosphodiesterase inhibitors, 4-(3,4-dialkoxyphenyl)2-pyrrolidones, has been found to be extremely potent with regard to potentiation of norepinephrine and adenosine-elicited accumulations of cyclic AMP in brain slices. The pyrrolidone like the less active 3-(3,4-dialkoxybenzyl)-imidazolidinones appeared to inhibit rather selectively cyclic AMP and not cyclic GMP-phosphodiesterases. Inhibition of calcium-dependent cyclic AMP-phosphodiesterase activity by the pyrrolidone and the imidazolidinone appeared to correlate with their potency in increasing accumulations of cyclic AMP in brain slices. The pyrrolidones because of the potency and apparent lack of effect on adenosine "release" would appear the agents of choice for study of cyclic AMP-phosphodiesterases in brain tissue. Isobutylmethylxanthine appears the most effective inhibitor of cyclic GMP-phosphodiesterases based on studies with brain slices. (Miyake, Ohga and Schwabe)

A calcium-dependent activator protein has been purified from rat brain to homogeniety and coupled to I) cyanogen bromide-activated Sepharose or II) N-hydroxysuccinimide-activated succinylated aminopropyl agarose. Affinity chromatography with I but not II led to an excellent separation of calcium-dependent phosphodiesterases from rat brain homogenates. (Creveling, Miyake)

Significance to Biomedical Research and the Program of the Institute: The key role of cyclases and cyclic AMP and cyclic GMP 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. Secondary effects due to elevated cyclic AMP levels in brain tissue will be investigated. The in vivo effects of drugs and the effects of lesions and hormonal alterations on the responsiveness of the cyclic AMP systems will be further explored. The nature of the adenosine receptor will be studied further using adenosine analogs modified for possible affinity labeling. Other agents with selective effects on adrenergic, serotonergic and histaminergic systems will be sought. The role of calcium and other ions in the function of cyclic AMP-generating systems will be further investigated. Correlations between cyclase responses and behavioral activity will be studied in crosses between rat and mouse strains. The effect of viral alteration of cerebellar development on cyclase responses will be probed. Correlations between cyclic

GMP and cyclic AMP levels in brain slices will be attempted. Antibodies to enzymes and regulatory proteins involved in the cyclic AMP-generating systems will be prepared and effects of various regimens on the turnover, levels and distribution of these enzymes and regulatory proteins in brain will be investigated. Further attempts to develop more satisfactory inhibitors of cyclases, phosphodiesterases and kinases will be made.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 20200-08 LC

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

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

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.5	6.5	0

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

Our primary goal has been the elucidation of the reactive metabolite which is responsible for the carcinogenic activity of the environmental contaminant benzo[a]pyrene. The approach taken consists of: i) synthesis of all possible primary oxidative metabolites as well as selected secondary oxidative metabolites, ii) study of the metabolism of benzo[a]pyrene with liver microsomes, as well as purified and reconstituted cytochrome P-450 systems with and without epoxide hydrase, iii) tests for inherent mutagenicity of the synthetic metabolites toward bacterial and mammalian cells, iv) elucidation of the roles of the cytochrome P-450 system and epoxide hydrase in potentiating or obliterating the mutagenicity of these metabolites, v) determination of the carcinogenic activity by topical application and subcutaneous injection for these compounds, vi) determination of the rate and nature of the products formed when reactive metabolites such as arene oxides and diol epoxides react with biopolymers and less complex chemical analogs.

Project Description:

Objectives: To study the basic mechanisms of enzymatic oxygen activation and incorportion into organic substrates. To explore enzymatic and nonenzymatic reactions of labile intermediates such as epoxides and peroxides. To devise methods for the synthesis and study of the chemical reactions of arene oxides. To investigate the role of labile oxygenated intermediates in the cytotoxicity and carcinogenicity of xenobiotic substances.

Methods Employed: Isotopically labeled substrates, water, and oxygen gas have been used to study the oxidation of organic compounds to labile metabolites by a variety of enzymes. Nuclear magnetic resonance spectroscopy, mass spectrometry, x-ray crystaliography and other physico-chemical techniques have been employed frequently in these studies. In addition to the usual techniques employed for the separation of metabolites, high pressure liquid chromatography is being pioneered in this area. Mutagenesis is studied in bacterial and mammalian cells and correlated with whole animal carcinogenicity.

Major Findings: In order to pursue the metabolism, toxicity, and carcinogenicity of hazardous substances, it has been necessary to synthesize and study the properties of a wide variety of potential metabolites. Previous reports have described methods for the synthesis of K-region and non-K-region arene oxides of polycyclic aromatic hydrocarbons. Binding of these arene oxides to nucleic acid and poly(G), potential macromolecular targets for mutagenesis and carcinogenesis has been studied. Both uv spectra and fluorescence have been used to estimate the extent of covalent interaction. The major conclusions of this study are that the size of the hydrocarbon and the type of arene oxide are very important factors. Stable K-region arene oxides bind more extensively than non-K-region arene oxides. The extent of this binding is directly related to the size of the hydrocarbon, possibly due to intercalation, but is not necessarily related to the carcinogenicity of the parent hydrocarbon. (Dansette and Yagi).

A major effort aimed at the synthesis of all twelve of the possible phenolic metabolites has now been completed. The general procedure for the final step in these syntheses has been either the dehydrogenation of ketones or Friedel-Craft cyclization of aryl acetic acids. In most cases, numerous prior synthetic steps were required to reach the actual precursors of these phenols. One year carcinogenicity tests on mouse skin are in progress with these compounds. (Yagi, Hernandez, Holder, Yeh, Dansette).

Since bifunctional chemical agents which are capable of cross-linking nucleic acid and protein are known to have toxic and carcinogenic activity, a synthesis of a bis-arene oxide was devised. In this procedure, both K-regions of pyrene were cleaved to dialdehydes and then cyclized to epoxides. The resultant 4,5:9,10-bis-oxide of pyrene was found to be inactive as a mutagen and no more than weakly toxic. (Moriarity and Dansette).

Preparation of the long sought after 7,8-diol-9,10-epoxide stereoisomers of benzo[a]pyrene has finally been achieved. The trans 7,8-dihydrodiol can be

directly epoxidized to the diol epoxide in which the 7-hydroxy group and the epoxide are on opposite faces of the molecule. Cyclization of a halohydrin derived from the diol produces the other isomer in which the 7-hydroxy group and the epoxide are on the same face of the molecule and can intramolecularly hydrogen bond. This molecular feature accounts for the remarkable 500-fold greater reactivity of this isomer toward simple nucleophiles. Extremely high reactivity (half-life of 30 sec in aqueous media for the more reactive isomer) of these diol-epoxides accounts for the difficulty encountered in their synthesis. (Yagi and Hernandez).

Continued studies on the metabolism of the environmental carcinogen benzo[a]pyrene have shown that the standard fluorimetric assay for enzymatic oxidation of this compound greatly underestimates the amount of phenolic products formed. Determination of the fluorescence yield of metabolites in acid media where the fluorescence of the twelve possible phenols is similar (in contrast to base where they differ by 10^3) indicates a 50% higher turnover number for the system. This study refutes the widely held view that 3-hydroxy-benzo[a]pyrene is the sole if not preponderant phenolic metabolite. (Holder, Yagi, Levin, Lu).

Profiles of metabolites from benzo[a]pyrene, produced by liver microsomes from genetically different strains of mice, have been analyzed with the aid of high pressure liquid chromatography. Previously, these mice had been classed as "responsive" (C57/BL6) or "nonresponsive" (DBA/2J) depending upon whether benzo[a]pyrene hydroxylase was inducible in the liver with 3-methylcholanthrene. They also differ in their susceptibility to carcinogens. The analysis established that i) the C57/BL6 mouse is inducible while the DBA/2J mouse is not even when compared on an individual metabolite level, ii) mouse liver differs from rat liver in that the ratio of epoxide hydrase to the cytochrome P-450 system in the rat is much higher than in the mouse as evidenced by the low levels of dihydrodiols produced by the mouse, and iii) that all primary oxidative metabolites including phenols, quinones, and dihydrodiols are subject to extensive secondary oxidative metabolism under conditions of low substrate concentration, high protein concentration, and long incubation times. Under the latter incubation conditions, substantial amounts (>30%) of metabolites are formed which are no longer extractable into organic solvent, possibly due to convalent binding to components of the incubation medium. (Levin, Conney, Holder, Yagi).

Reconstitution of the microsomal enzyme system for drug metabolism has taken an important step forward with the purification of epoxide hydrase. The homogeneous preparation was obtained from an ammonium sulfate precipitate of solubilized microsomes after several column chromatographies in the presence of detergents. (Lu, Levin, Daly).

Biological testing of the synthetic benzo[a]pyrene metabolites is now in full progress, and substantial information is presently available. Arene oxides and the corresponding phenols at the 4,5-, 7,8-, and 9,10-positions have been examined for inherent mutagenic activity with bacterial and mammalian cells which lack the capability for drug metabolism.

In both test systems, the six phenols were either weak or inactive. High mutagenic activity was observed for the 4,5- K-region arene oxide while the two non-K-region arene oxides were very weak as mutagens. Carcinogenicity studies on mouse skin showed a different pattern of activity in that the 9,10- and 4,5- oxides were inactive while the 7,8-oxide was a potent carcinogen, although weaker than benzo[a]pyrene. Evidence has been forthcoming which indicated that the 7,8-oxide may not have inherent carcinogenic activity but may be transformed by epoxide hydrase and then the cytochrome P-450 system into the stereoisomers of the 7,8-diol-9,10-epoxide.

The substantial synthetic effort which has been invested in the diol epoxides has proved quite rewarding in that they are among the most mutagenic compounds yet tested with the Chinese hamster V-79 cell line. The stereoisomer with the benzylic hydroxy group on the same face of the molecule as the epoxide ring is 20-fold more mutagenic in the bacterial tests and >40-fold more mutagenic in the mammalian cells when compared to the very mutagenic benzo[a]pyrene 4,5-oxide. Results to this point are highly indicative that the 7,8-diol-9,10-epoxides may be the ultimate carcinogens of the hydrocarbon.

Significance to Biomedical Research and the Program of the Institute: Although enzymatic oxidation and drug metabolism had long been held to constitute a means of detoxication, results of the type described here make it quite clear that certain of these pathways for drugs and environmental chemicals result in toxic and carcinogenic metabolites. Once the nature of these active metabolites have been identified, it may become clear as to which industrial and agricultural chemicals are unsafe for use. The potential impact on the design of safe drugs is clear.

Proposed Course of Project: The major impetus for the coming year will focus on the benzo ring of benzo[a]pyrene since the present results indicate that the ultimate carcinogen probably involves this portion of the molecule. Should the results of these studies continue to indicate a key role for diol epoxides, selected metabolites from other polycyclic hydrocarbon carcinogens will be prepared to further test this theory. A substantial effort will be directed toward establishing the kinetic properties of homogeneous epoxide hydrase and glutathione S-transferases toward arene oxides from several hydrocarbons.

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ANNUAL REPORT
LABORATORY OF EXPERIMENTAL PATHOLOGY

Studies in the Section of Molecular Pathology (Dr. George G. Glenner and staff) have continued to be concerned with various aspects of the pathogenesis of Amyloidosis.

Human Secondary Amyloidosis: Amyloid of unknown origin (AUO) is the designation for a class of amyloid fibrils deposited in a form of systemic amyloidosis which occurs in man and other classes of animals and which is distinct from human amyloidosis of immunoglobulin origin. The major protein contained in these fibrils is called AA and its amino acid sequence does not correspond to that of any known protein. Since the detection, in serum, of a component called SAA with antibodies raised to denatured amyloid A fibrils, there has been considerable interest in the isolation of SAA both for biochemical studies of its role as a possible precursor of the AA protein deposited in tissues, and for use as an immunogen for immunohistochemical studies to identify the cell of origin of SAA. Work carried out in several laboratories, including this one, has shown that SAA has the properties of an acute phase reactant in man, mink, and mouse, and it has been reported that murine SAA has an immunosuppressive effect. To date, the only form of SAA which has been purified is a polypeptide chain of 12,500 m.w., SAAL, which has been isolated from serum by gel filtration in guanidine HCl or 10% formic acid, or electrophoresis on SDS polyacrylamide gels.

The native structure of SAA is unknown; it is possible that SAAL is complexed through noncovalent bonds to a high m.w. polypeptide which does not cross-react with anti-AA antibodies, or that SAA is a polymer of SAAL polypeptide chains arranged in an unknown fashion, perhaps containing additional micromolecular material such as peptides or lipids. One experimental approach to determine whether there was a high molecular weight polypeptide carrier for the SAAL polypeptide, was to radiolabel the SAA fraction from whole serum with iodine 125 and to then mix it with unlabeled, whole, SAA containing serum and carry out immunoprecipitation with anti-AA antibodies using a double antibody technique. The immunoprecipitated ^{125}I-SAA was analyzed by SDS urea-polyacrylamide gel electrophoresis: only a fraction of the precipitated radioactivity was observed to migrate with a m.w. of 12,500. Multiple radiolabeled species and molecular weights intermediate to SAA and SAAL were also detected and appear to represent incompletely dissociated SAA, suggesting that SAA is an aggregate of several SAAL chains.

The isolation of SAA was greatly hampered by the lack of a quantitative assay. During the past year, a solid phase radioimmunoassay for AA, which employs purified antibodies to AAE (the major fraction purified from AA by isoelectric focusing) adsorbed onto the wells of flexible microtitration plates, was applied to the purification of SAA from serum. In addition to facilitating the isolation procedure, the use of the RIA for monitoring the fractionation of SAA-containing serum has revealed information about the conformation and flexibility of SAA.

Several properties of SAA suggest that in its native conformation the antigenic determinants which cross-react with antibodies raised to AA are relatively inaccessible or buried. The native molecular weight of SAA appears to be approximately 160,000 as determined by gel filtration of fresh serum in PBS. SAA appears to be labile in that it both aggregates and dissociates upon storage or upon usually nondissociating manipulations. There is an increase in immunoreactivity of SAA-containing serum upon storage and experimental manipulation which does not normally dissociate noncovalent bonds as well upon isolation of SAAL by gel filtration under dissociating conditions. The increase relative to whole serum is two fold for the isolation of SAA by gel filtration four fold for the isolation of guanidine HCl denatured SAAL and almost 150-fold for the isolation of SAAL in the presence of formic acid. RIA determination of SAA in whole serum can reach levels of 5 µg/ml of AA cross-reactivity, yet sera with cross-reactivity as low as 50 mg/ml are strongly reactive in double immunodiffusion. It appears that unfolding of cross-reacting determinants occurs during the process of double immunodiffusion.

Qualitative immunodiffusion assays were compared with RIA for SAA. Except in sera from individuals with multiple myeloma, higher levels of SAA correlated with positive immunodiffusion assays. Ninety-three percent of the pathological sera with SAA levels greater than 100 ng/ml had positive immunodiffusion tests. For approximately 150 normal sera a median value of 8 ng/ml SAA was observed with no age related increase in subjects ranging in age from 16 to 70 years. SAA levels tended to be high in certain categories of neoplastic, inflammatory and infectious diseases, as well as in secondary amyloidosis. Less elevation was observed in primary amyloidosis. The results are consistent with many recent observations that SAA levels fluctuate like acute phase reactants, are in essential agreement with a previously reported study by Rosenthal and Franklin which employed an RIA different in principle from the one used in this laboratory. The RIA for SAA has no prognostic or diagnostic significance for secondary amyloidosis at the present time. (Drs. Sipe, McAdam and Glenner).

Primary Structure of a Familial Amyloid Protein: To date, two classes of systemic amyloid proteins have been chemically characterized: amyloid of immunoglobulin origin and amyloid A of unknown origin. Costa and coworkers have identified a third class of protein deposited systemically in a heredofamilial form of amyloidosis. Our immunochemical studies have shown that the major protein solubilized from these deposits cross-reacts with antibodies raised to a preparation of normal human prealbumin, the chemical purity of which has been established by amino acid sequence determination of the first thirty residues from the amino terminus. We have established in collaboration with Waxdal and coworkers a microsequencing methodology employing the Beckman spinning cup sequencer, ^{35}S-PITC, and thin layer chromatography with autoradiographic determination which can identify at least thirty residues using approximately 50 nmoles of protein. Preliminary sequence data show both sequence similarities and some structural differences between the familial amyloid protein and the normal prealbumin (Drs. Sipe and Costa).

The studies in Section on Chemical Pathology (Dr. Friedman and staff) have been concerned with various aspects of cell-virus interactions.

Studies on SV40 early antigen: Whether a specific viral infection will lead to virus production (lytic infection) or to cell transformation (oncogenesis) depends upon the interactions between virus-specific macromolecules, either carried by the invading virus or coded for by the virus shortly upon infection, with macromolecules specified by the host cell. The study of the synthesis and function of the early SV40 antigens has been complicated by the fact that the amount of viral proteins made early post-infection is quantitatively very low, compared to the extent of host macromolecular biosynthesis. To overcome this problem, a series of Ad2-SV40 hybrid viruses have been used, each containing the entire Ad2 genome covalently-attached to varying amounts of SV40 DNA, which have the ability to turn off the host translational activity upon infection, to identify the various SV40-specific early functions. Each of these viral polypeptides have been isolated, biochemically-characterized, and localized to specific subcellular fractions or functional sites. A search is now being made for any biochemical change that may accompany the appearance of a specific viral antigen upon virus infection, and with the data obtained from the localization studies, designing cell-free systems to probe the functions of specific antigens. (Drs. F.T. Jay and G. Jay (NCI)).

Studies on interferon inhibition of murine leukemia virus replication: It has been previously established in this and other laboratories that interferon inhibits oncornavirus production at a late stage of virus assembly or release. Morphologically, the accumulation of budding virions on the surface of interferon treated cells resembled a similar late block in virus maturation in a spontaneous temperature sensitive mutant (ts-3) of Moloney MLV. Studies have been performed to examine the temporal relationship between these two defects. Cells chronically infected with MLV-ts-3 grown at the non-permissive temperature accumulate budding virions on the cell surface. The effect of interferon treatment was examined in a time course of virus release after shift-down to the permissive temperature. A variety of experimental techniques including viral assay by reverse transcriptase activity, viral infectivity, intracellular gs antigen assay and electron microscopic observation all consistently substantiated the fact that the interferon sensitive step preceeds the temperature defect.

Preliminary data also suggested that interferon treatment may result in the formation of defective viral particles. Wild type MLV grown at the non-permissive temperature in the presence of interferon released large numbers of virus particles detected by enzyme assay that were not detected in infectivity assays. Since published data on these temperature sensitive mutants had not taken into account the effect of elevated temperature on the stability of viral enzyme activity or infectivity, experiments are being performed to clarify these effects. Once the kinetics of both virus production and degradation are established at both permissive and non-permissive temperatures, the effect of interferon can more accurately be evaluated. Electron micrographic observations

are being made to correlate with data concerning the biologic activity of these particles. A detailed biochemical analysis of the viral RNA and proteins will be used to support the definition of the physical nature of this "defectiveness". (Drs. Myers, Chang, Friedman, Wong (Univ. of Illinois)).

Structural analysis of AAV DNA and transcription map of AAV genome: A total of about 80 specific fragments of AAV DNA produced by 10 different restriction endonucleases have been mapped. These fragments have been used for a variety of studies. Strand separation of the terminal fragments of AAV DNA produced by R. EcoR1 allows definition of the DNA strand polarity and direction of RNA transcription in relation to the restriction fragment maps. The maximum and minimum estimates of the size of the terminal repetitions in AAV DNA are 120 and 40 nucleotides respectively which are equivalent to 3% and 1% of the genome length. Some of the duplex termini of AAV DNA contain a non-linear ("rabbit ear") structure which is interpreted as evidence for the presence of terminal palindromes which show both reiteration and variation in sequence. These fragments have been used to accurately map the portion of the genome represented in stable AAV mRNA. This map provides a basis to design further experiments to locate the putative specific promoter for AAV RNA transcription. (Drs. Carter, de la Maza, Jay, Khoury (NCI), Denhardt (McGill) and Berns (Johns Hopkins)).

Studies on the biochemical changes in the plasma membrane of interferon treated cells: Preliminary studies on the isolated plasma membrane of mouse AKR, C^+ cells showed that there are at least two fractions of membrane separated by equilibrium centrifugation on the discontinuous sucrose density gradient. In membrane preparations of control AKR, C^+ cells, the low density component constitutes about 75% (A_{250}) of the two fractions. However, if the cells were treated with interferon for 16-24 hr. prior to membrane preparation, the distribution of the two density component is reversed. Similar results have also been observed in AKR, C^- cells which do not normally produce virus. The increase in the proportion of the higher density plasma membrane component would therefore seem to be a cellular response to the interferon treatment and the membrane may be the site of interferon action.

Additional studies employing the freeze fracture technique to define morpologic atterations in membranes indicate that interferon treated AKR cells have a significant increase in the number of intra-membraneous granules.

Studies with cholera toxin indicate that interferon treatment causes complex changes in the binding pattern of cholera toxin to L cell plasma membranes.

All of these studies suggest that interferon may directly cause changes in the plasma membrane of animal cells. (Drs. Jay, Chang, Friedman and Kohn (LBP)).

Interactions of biological and chemical activities of interferon, cholera toxin, and thyroid stimulating hormone (TSH): Cholera toxin (10^{-9} M) or TSH (10^{-5} M) inhibit the establishment of antiviral activity when added with mouse interferon to the medium of Ly cells. In the case of cholera toxin addition of the toxin before interferon also inhibits development of antiviral activity. Neither TSH nor toxin affects already established antiviral activity.

On the other hand, interferon treatment of L cell membranes inhibits binding of both cholera toxin and TSH. These studies may allow us to define how much of an interferon's species specificity is due to its failure to bind to cells in which it is not active. They may also help us to develop simple biochemical assays for interferon. (Drs. Friedman and Kohn (LBP)).

Interferon and Chronic Virus Infections? In Ly cells treated with interferon and then infected with high multiplicities of vesicular stomatitis virus a prolonged viral infection is initiated. In this system virus production persists from 14 to more than 26 days. This chronic infection seems more likely due to endogenous interferon generated by the virus infection than to generation of defective interfering virus forms. (Ramseur (doctoral thesis project) and Dr. Friedman).

The following studies have been conducted in the Section on Cytogenetics (Dr. Tjio and staff) during the past year.

Cytogenetics of Hematopoetic Disorders: These studies are being carried out by our section chief, Dr. Joe-Hin Tjio, while on sabbatical at the University of Claude-Bernard in Lyon, France. He and Dr. D. Germain are utilizing newly-developed differential staining methods to analyze abnormal hematopoetic cells found in patients with hemopathies such as Fanconi's anemia and in patients exposed to leukemogenic substances such as benzene and related polycyclic hydrocarbons. They are attempting to find specific chromosomal defects in direct marrow preparations as well as in cultures established from hematopoetic tissues. They are also conducting similar studies on mice as a model for the human studies. Since some of the techniques for differential staining of chromosomes being used in France are different than those commonly used in our laboratory, Dr. Tjio is becoming familiar with these and will be utilizing them here after his return. (Drs. Tjio and Germain).

Murine Robertsonian Translocations: During the year, eight different translocation lines derived in our laboratory from mouse Robertsonian translocations Rb(5.19), Rb(9.19), Rb(6.15), and Rb(8.17) have been maintained. All are maintained individually in the homozygous condition, and several stocks are homozygous for more than one translocation. The most well-characterized stock, Rb(5.19) 1WH, is currently in generation 36 of inbreeding; it has recently been utilized for cleft palate studies by investigators outside of the NIH and may prove to be one of the best stocks in existence for cleft palate induction experiments. We are continuing to characterize our newly-established strain, Rb(5.19)/Rb(6.15)/ Rb(8.17), with a chromosome number of 34 (normal = 40). It remains fertile, has a low rate of meiotic

nondisjunction when homozygous (<1%) and a high rate when heterozygous (12.0% trisomic embryos at 10 days gestation). Studies of this strain are continued. At present, investigators both at and outside of the NIH have expressed interest in using these strains for tumor research and for production of allophenic mice, since they have marker chromosomes not carried by normal mice (Dr. White, Crandall and Raveche).

New Differential Staining Techniques: Two techniques have been added to those already in use in our laboratory: Sister Chromatid Exchange (S.C.E.) is a phenomenon noted in Giemsa-stained metaphases when cells are cultured in the presence of bromodeoxyuridine (BUdR), stained with the benzimidazole derivative Hoechst 33258, and treated with ultra-violet light. Metaphases from normal individuals have only a small number of S.C.E.'s per cell following this treatment. In Bloom's syndrome, an autosomal recessive disease characterized by high chromosome breakage rates, the S.C.E. rate has been found by others to be tremendously increased. Investigations by others also indicate that the exchange rate is increased by alkylating agents in a dose-related fashion, and that S.C.E. is a more sensitive indicator of chromosome damage than traditional morphological methods. At present, the normal control rate of exchanges in human lymphocyte cultures are determined and have been studied in several patients with this technique. The method is also being used for the studies of several mouse strains and will continue to be utilized for studies of patients with suspected exposure to mutagens or possible chromosomal breakage syndromes.

Replication Sequences with Differential Staining: The addition of thymidine (TdR) to cells growing in a medium containing 5-bromodeoxyuridine (BUdR) at the end of the first DNA replication cycle (S phase) results in the incorporation of TdR into late replicating chromosomal regions. These sites can be visualized by staining metaphase chromosomes with the same 33258 Hoechst-giemsa procedure that is utilized for the S.C.E. technique. The early replicating sites of BUdR incorporation stain less intensely with Hoechst-giemsa than the late replicating regions that have incorporated TdR, making it possible to determine the time during the S period that specific chromosomal bands and segments replicate. The reverse of this method involves the addition of BUdR during the last few hours of the S phase. Late replicating regions can be visualized with this variation of the techniques as light staining regions. The banding patterns seen following both methods of treatment agree with sequences of replication demonstrated by standard autoradiographic techniques, but are more accurate and precise. These techniques are now being used to study patients with abnormal sex chromosomes (isochromosome X and non-fluorescent Y), and are also necessary when other banding methods (C-, G-, and Q-banding) cannot permit identification of abnormal chromosomes. (Dr. White and Raveche).

Short Stature: The study of frequency of sex chromosome defects in pre-pubertal and teen-aged girls with short stature have been continued in collaboration with Dr. R. Johnsonbaugh (NNMC, Pediatric Endocrinology). During the first year, 4 of 13 cases had abnormal karyotypes (30.8%). This past year, 4 of 14 cases were abnormal (28.6%), supporting our

initial impression that the population of short girls with parents of average or above average stature has a very high frequency of sex chromosome defects such as 45,XO, 45,XO/46,XX, or 45,XO/46,XY. This survey will be continued during the coming year, and several of the more unusual patients will be described in case reports. As an outgrowth of the short stature study, Dr. Johnsonbaugh has initiated a study with our laboratory and the NNMC Pediatric Cardiology group to determine the frequency of sex chromosome defects in females with left-sided congenital heart disease. All of six cases completed so far have had normal karyotypes (Dr. White and Crandall).

Chromosomal Banding Analysis of Patients with Congenital Defects or Syndromes and Genetic Diseases: During the year, a total of 63 cases were studied; 23 from NNMC and 40 from various institutes at the NIH. Twelve cases of Kallman's syndrome (hypogonadotropic hypogonadism) from NIAMDD were of special interest. The banded karyotypes of five females and seven males with the syndrome were normal, a finding consistent with other evidence that it is an autosomal recessive disorder rather than a syndrome associated with a visible chromosome defect. Dermatoglyphic analysis is also being done on this group of patients. The remaining 28 consultations from NIH were predominantly syndromes of unknown etiology and typical cases of gonadal dysgenesis, all requiring banding rather than routine karyotyping. Of this latter group, three cases of pericentric inversion for chromosome number 9, four cases of Klinefelter's syndrome, and five cases of low grade mosaicism for sex chromosome aneuploidy were detected. Of all 63 cases studied this past year, 20 were abnormal (31.8%), reflecting our selection of only those cases of research interest. (Dr. White, Crandall and Raveche).

Murine Tumors: During the past year, collaborateive studies with Dr. W. Rowe (NIAID) in chromosomal analysis of mouse thymomas have been done. Others have reported specific trisomy (chromosome 15) in thymomas for AKR mice, and the attempt is made to determine with banding techniques the karyotypes of thymic tumors of AKR hybrids carrying specific isolated genetic loci known to be related to leukemogenesis. After five experiments, it has been found that the tumors fall into two groups; those with non-specific chromosomal abnormalities, and those which are normal. It is too soon to determine if the karyotypic findings are related to specific loci, and this study will be continued during the coming year. (Dr. White).

Primate Chromosomes: Collaborative studies with Dr. W. Caveness (NINDS) in chromosome studies of tissue cultures from irradiated Rhesus brain are continued. Our co-investigators are attempting to clarify the mechanism of endothelial cell proliferation seen after radiation-induced brain damage with biochemical, viral, and pathological techniques. At present, the attempt is made to improve growth of brain tissue by using selective media, since poor growth of damaged as well as normal tissues has limited most of the past experiments. We are also continuing to collaborate studies with Dr. D. Symmes (NICHD) in karyotypic studies of a series of chromosomal inversions in squirrel monkeys are continued. This is

a very long term study attempting to correlate behavioral and genetic factors, and may provide a primate model for inversion effects in humans. (Dr. White).

Murine Immunogenetic Studies: Experiments are currently being performed in collaboration with Dr. A. Steinberg (NIAMDD) to determine the effect of the NZB genetic background on the immunological status of NZB/DBA F_1 hybrid mice. The NZB strain, characterized by immunological abnormalities of the autoimmune type, has naturally occurring thymocyte antibodies. We have detected no such antibodies in NZB/DBA F_1 females normally produce these thymocyte antibodies, and removal of the ovaries does not alter this response. Cytogenetic studies, including sister chromatid exchange, are currently being performed on these mice. It is hoped that the immunological and cytogenetic findings can be correlated. (Raveche and Dr. Steinberg (ARB)).

The studies in the Section on Biophysical Histology (Dr. Feder and staff) on histochemical and ultrastructural studies: methods and applications.

Beta-glucuronidase as a Cell Marker: Studies have been concluded on chimeric (tetraparental) mice with β-glucuronidase as a cell marker. The chimeric mice were made from pairs of inbred mouse strains whose tissues are known to differ in β-glucuronidase activity. Thus the mosaic tissues of the chimeric mice were composed of two intermingled populations of cells which could be distinguished microscopically in tissue sections stained histochemically for β-glucuronidase. (a) In many of the mosaic tissues there were small clusters of cells (i.e., cells of one staining type -- say high-glucuronidase cells -- surrounded by cells of the contrasting type). Small clusters of cells and solitary cells were found in a wide range of tissues, primarily epithelial tissues. This finding contrasts with several reports by other investigators that mosaic tissues are composed of large clusters -- in the range of 10^4 to 10^8 cells per cluster. The presence of small clusters and solitary cells supports but does not prove the conclusion that cell migration may continue in many tissues throughout their growth. (b) Staining differences between cells of chimeric mice and cells of the same tissues in single-genotype control mice showed some interesting regularities consistent with the hypothesis that there is an exchange of enzyme between cells. This hypothesis receives support from the preliminary finding that some hepatocytes of chimeric mice contain enzyme characteristic of both the high-glucuronidase and low-glucuronidase strains of mice. These observations, together with reports by other investigators that exogenous lysosomal enzymes are taken up by cells in the intact animal and in cell culture, support the view that exchange of glucuronidase (and other enzymes) among cells actually occurs as a normal function in a wide range of cell types. (Drs. Feder, Herrup (Harvard Med. School), Mullen (Harvard) and Sidman (Harvard)).

Marking Neurons for Light and Electron Microscopy: Studies with two highly fluorescent tissue-reactive dyes (N-substituted naphthalimides) have been concluded. The dyes, which are now synthesized on a large

scale and in high yield, are analytically pure. (a) These dyes have been used to mark individual neurons in vivo through recording microelectrodes. Successful marking of a wide variety of vertebrate and invertebrate neurons has now been carried out in laboratories at the N.I.H. and elsewhere. (b) The occurrence of clusters of coupled neurons has been confirmed by injection of a single neuron and observing spread of the dye to contiguous neurons. (c) By injection of dye into the eye of the chick embryo, it has been possible to demonstrate reverse axonal transport along the optic nerve to the isthmo-optic nucleus. Two further studies are in progress. (d) The naphthalimide dyes are under investigation as labels of γ-globulin and other proteins. The high fluorescence efficiency of the dye and certain other properties make it promising as a label for proteins. (e) Studies on marking of single neurons for electron microscopy with heavy metals are in progress. These studies have until now been unsuccessful. (Dr. Feder, Stewart, Drs. Bertrand (NINCDS), Detwiler (NINCDS), Fuortes (NINCDS) and LaVail (Harvard)).

Observations on structures in thyroid which metabolize catecholamines (Dr. Tice).

Junctional Development in Fetal Thyroid: Continuing a series of studies on the thyroid gland with a combination of electron microscope techniques, developmental changes in fetal thyroid tight junctions have been examined. Using colloidal lanthanum as a tracer, it was found that although tight junctions are present in the developing follicles of 16-17 day fetal rats, they do not impede the passage of La from the extracellular space into the lumen of the developing follicle until about 20 days of gestation. When fetal thyroid tight junctions are examined using freeze-fracture methods, the earliest follicular lumens examined always are surrounded by a continuous, although sometimes rudimentary, tight junction meshwork. La appears to reach the follicular lumen by leaking around the elements of the tight junction via small discontinuities (40-120 Å) in the elements. These findings can be related 1) to the role of the tight junction, retaining thyroglobulin in the follicular lumen once its synthesis has begun, and 2) the development of transepithelial potentials in the gland, for the intercellular space acts as a paracellular shunt, tending to reduce such potentials if its resistance is low. (Dr. Tice).

Immunocytochemical Distribution of Catecholamine-O-Methyl Transferase (COMT): The distribution of COMT has been examined in several rat tissues using an indirect labelling technique. In liver, biochemically and cytochemically, the enzyme appears to have a microsomal distribution. Studies are currently in progress in an attempt to validate this localization, to determine the contribution (or lack of it) of soluble COMT to the staining patterns observed, and to obtain conditions suitable for electron microscopic localization. (Drs. Inoue and Creveling (LC)).

Synovial Mucin Lubrication: Ellipsometric measurements at National Bureau of Standard (NBS) of hyaluranate-protein films on silica briefly mentioned in last year's summary have been refined and completed. The values obtained are consistent with earlier determinations by other methods, and the NBS experiments have added information about the solids

content of the adsorbed gel layer.

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Theoretical calculations indicate that an important part of the boundary lubrication can arise from repulsive electrostatic floatation of one articular cartilage surface approximately 100 Å away from its mating surface. If this proves to be correct, understanding the general biological boundary lubrication mechanism for the viscera as well as for the skeletal joints should profit from the pre-existing mathematical development of colloid stability theory, which includes both electrostatic and ateric or osmotic components, the latter having been treated earlier by Dr. McCutchen.

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McCutchen has been asked to write a chapter on joint lubrication for "Joints and Synovial Fluid," edited by L. Sokoloff. This has required reading the literature and understanding theoretical treatments of cartilage and synovial fluid behavior. (Dr. McCutchen).

Studies Related to the Naturally Occurring Carcinoges, Cycasin and its Metabolities: The past year's effort was largely devoted to the writing of a summarying report on the carcinogenicity, mutagenicity and teratogenicity of cycasin, a β-glucoside, of methyl-azoxymethanol (MAM), the proximate carcinogen of cycasin and of the synthetic MAM acetate. With this general review, the cycasin studies started in 1961 have come to an end in this laboratory. In collaboration with E.C. McDaniel (LNE-NIAMDD), two of the precursor in the pathway of synthesizing MAM acetate, namely dimethylhydrazine and azoxymethane were administered by gavage to germ-free Sprague-Dawley rats. It was found that both compounds induced cancers of small and large intestine. The number of cancers per germfree rat increased with the number of weekly intragastric administration. The earliest cancer was found 3 months after the first dose of dimethylhydrazine had been given (Dr. Laqueur).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,000-03 LEP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies related to oncogenicity of methyazoxymethanol.

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

PI:	G. Laqueur	Chief, Lab. of Exptl. Path.	LEP:NIAMDD
OTHER:	E. McDaniel	H.S. Director	LNE:NIAMDD
	R. Yamamoto	Research Chemist	CMT:NCI

COOPERATING UNITS (if any)

NCI-EXP. (Dr. R. Yamamoto)
LNE-NIAMDD (Mr. McDaniel)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

None

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5-3/4	1	4-3/4

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

Methyazoxymethanol has been synthesized as the acetate ester from dimethylhydrazine with an intermediate azoxymethane. Both precursors are highly carlindgenic for the intestines of germfree rats.

PHS-6040
(12-75)

Project Description:

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Publications:

Laqueur, G.L.: Multiple primary tumors induced with the glycoside cycasin and its aglycone. The 5th Multiple Primary Malignant Tumor Conference on Cancer. Perugia, Italy. April 1973. p. 919, 1975.

Laqueur, G.L. and Spatz, M.: Oncogenicity of cycasin and methylazoxymethanol (MAM). In Odashima, S., Sato, H. and Takayama, S. (Eds.): Recent Topics in Chemical Carcinogenesis. Gann Monograph on Cancer Research. Tokyo, Japan. University of Tokyo Press. 17:189, 1975.

Hollander, C.F., Burek, J.D., Boorman, G.A., Snell, K.C., Laqueur, G.L.: Granular cell tumors of the central nervous system of the rat. Arch. of Path. In press.

Laqueur, G.L.: Environmental carcinogenesis in the Advances in Modern Toxicology, Vol. 2. Hemisphere Publishing Corp., Washington, D.C. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 21,001-02 LEP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Preparation of stained tissue sections for investigation and diagnostic purposes.

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

PI: V. Reveal Head,Tissue Preparation Lab. LEP:NIAMDD

OTHER: None

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Tissue Preparation Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/4	0	1

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

The tissue laboratory will continue to provide technical assistance to the investigators of the Laboratory of Experimental Pathology and to those of other units at the NIH who require technical help. A considerable part of the tissue laboratory's work is concerned with providing a wide variety of special histologic techniques adjusted to the various research projects as shown below.

	Specimen Accessioned	Stained Slides Routine	Stained Slides Special	Spare Slides	Total
Animals		693			
Surgicals		17			
		710	1308		
			2018	4772	
				6790	

PHS-6040 (12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,002-03 LEP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Immunocytochemistry of Catecholamine-O-Methyl Transferase (COMT)

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

PI:	L. Tice	Med. Officer (Research)	LEP:NIAMDD
OTHER:	K. Inoue	Visiting Fellow	LEP:NIAMDD

COOPERATING UNITS (if any)

Dr. C. Creveling LC-NIAMDD

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
None

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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

Methods has in situ localization of COMT in liver and brain are being evaluated, with particular attention to the contribution of soluble enzyme to the staining patterns observed.

Project Description:

Junctional Development in Fetal Thyroid: Continuing a series of studies on the thyroid gland with a combination of electron microscope techniques, developmental changes in fetal thyroid tight junctions have been examined. Using colloidal lanthanum as a tracer, it was found that although tight junctions are present in the developing follicles of 16-17 day fetal rats, they do not impede the passage of La from the extracellular space into the lumen of the developing follicle until about 20 days of gestation. When fetal thyroid tight junctions are examined using freeze-fracture methods, the earliest follicular lumens examined always are surrounded by a continuous, although sometimes rudimentary, tight junction meshwork. La appears to reach the follicular lumen by leaking around the elements of the tight junction via small discontinuities (40-120 Å) in the elements. These findings can be related 1) to the role of the tight junction, retaining thyroglobulin in the follicular lumen once its synthesis has begun, and 2) the development of transepithelial potentials in the gland, for the intercellular space acts as a paracellular shunt, tending to reduce such potentials if its resistance is low.

Immunocytochemical Distribution of Catecholamine-O-Methyl Transferase (COMT): The distribution of COMT has been examined in several rat tissues using an indirect labelling technique. In liver, biochemically and cytochemically, the enzyme appears to have a microsomal distribution. Studies are currently in progress in an attempt to validate this localization, to determine the contribution (or lack of it) of soluble COMT to the staining patterns observed, and to obtain conditions suitable for electron microscopic localization.

Publications:

Tice, L.W., Wollaman, S.H., and Carter, R.L.: Changes in tight junctions of thyroid epithelium with changes in thyroid activity. J. Cell Biol. 66:657-663, 1975.

Tice, L.W. and Creveling, C.R.: Electron microscopid identification of adrenergic nerve endings on thyroid epithelial cells. Endocrinology, 97:1123-1129, 1975.

Tice, L.W., Carter, R.L., and Wollman, S.H.: Gap junctions in thyroid epithelium of the rat. Endocrinology, 98:800-801, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 21, 003-02 LEP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biomechanics and related studies

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

PI:	C. McCutchen	Research Physicist	LEP:NIAMDD
OTHER:	J. Wilkins	Staff Fellow	LEP:NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
None

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/2	2	0

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

Fish wake photography was wound up reported to the conference on the Biodynamics of Animal Locomotion in Cambridge, England in Sept. 1976. The conclusions remain as described in the last annual report. The flow visualization method has been written up following solicitation from two journals, one biological, the other for physics teachers. Cone's theory of how birds avoid starting vortices by commencing wingbeats with wings touching has been examined theoretically to see if it anticipates Weis Fogh's clap-fling mechanism. In principle it can accomplish the same result, but Weis Fogh's mechanism is better mechanically. With Chopper technique a hearing aid can, in principle be 100% efficient. Battery drain is a serious problem with hearing aids. Allied to this is the idea of using a hearing aid-like device as an ear defender. To block acoustic transmission of sounds but pass sounds electronically, and play them at a safe loudness. A breadboard prototype has been built. Chatterbox, the vibration monitor for ultramicrotomes has been completed by Delhi Thweatt in improved form. To avoid accustic feedback it records the cutting sound on magnetic tape and waits until cutting is over and the amplifier turned off before playing the sound through a loudspeaker. A short article on the sociology of scholarly review may open discussion of a dark corner of science. The thesis

is that reviewing is a conspiracy evolved between (not plotted by) establishment and reviewers. It forces innovators to take their ideas to the establishment, which adopts or ignores them at its convenience. A chapter on joint lubrication is being written for "Joints and Synovial Fluid", edited by L. Sokoloff.

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Publications:

McCutchen, C.W.: Optical exxtent. Applied Optics 13:1537, 1974.

McCutchen, C.W.: Miniviscometer: A small couette instrument. Biorheology 11:265, 1974.

McCutchen, C.W.: Comment on "Bone articulations as systems of poroelastic bodies". AIAA Journal 12:256, 1974.

McCutchen, C.W.: Comment on "Some surface characteristics of articular cartilage I and II by V.C. Mow *et al*. *J. of Biomechanics* 8:261, 1975.

McCutchen, C.W.: Do mineral crystals stiffen bone by straight-jacketing its collagen. *J. Theor. Biology* 51:51, 1975.

McCutchen, C.W.: An approximate equation for weeping lubrication solved with an electrical analog. Report of Imperial College Conference on Articular Cartilage. *Ann. Rheum. Dis*. 34:85, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZOI AM 21,004 (09) LEP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Control of genetic function in interferon-treated and virus-infected animal cells

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

Dr. Robert M. Friedman	Chief, Section on Chemical Pathology	NIAMDD:LEP
Dr. Barrie J. Carter	Senior Staff Fellow	NIAMDD:LEP
Dr. Esther H. Chang	Visiting Fellow	NIAMDD:LEP
Dr. Maureen W. Myers	Staff Fellow	NIAMDD:LEP
Dr. Francis T. Jay	Visiting Fellow	NIAMDD:LEP
Dr. Luis M. de la Maza	Guest Worker	NCI
Mrs. Janet M. Ramseur	Research Biologist	NIAMDD:LEP

COOPERATING UNITS (if any) LBP:NIAMDD - L. Kohn, LP:NCI - A. Rabson, J. Costa and T. Triche, POB;NCI - G. Jay, LBV:NIAID - B. Moss, VBB:NCI - G. Khoury, McGill University - D. Denhardt, S. Eisenberg and K. Bartok, Johns Hopkins University - K. Berns and K. Fife, University of Illinois - P. Wong.

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Chemical Pathology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
11 1/2	7	4 1/2

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

We have employed several systems to investigate the fundamental problems of control of biological functions at the molecular level in animal cells. For this purpose we have been interested in virus replication and its natural inhibition since these can be studied intensively at the molecular level of organization. In the case of Adeno-associated virus we are investigating structure and function of what, in effect, is a single functioning gene. Early functions of an oncogenic agent, SV40 are also under study. These functions seem related to tumor induction.

In the case of interferon we have begun to elucidate the mechanism of action of this extremely potent, natural biological defense mechanism. We have found that interferon is active against murine leukemia viruses. It may initiate its action by altering plasma membranes. The binding of interferon to cells and the subsequent activation of cell control mechanisms are under intense study at present. Finally, we are also looking at interferon's complex role in chronic virus infections.

PHS-6040
(12-75)

Project Description:

1. Studies on interferon inhibition of murine leukemia virus replication: It has been previously established in this and other laboratories that interferon inhibits oncornavirus production at a late stage of virus assembly or release. Morphologically, the accumulation of budding virions on the surface of interferon treated cells resembled a similar late block in virus maturation in a spontaneous temperature sensitive mutant (ts-3) of Moloney MLV. Studies have been performed to examine the temporal relationship between these two defects. Cells chronically infected with MLV-ts-3 grown at the non-permissive temperature accumulate budding virions on the cell surface. The effect of interferon treatment was examined in a time course of virus release after shift-down to the permissive temperature. A variety of experimental techniques including viral assay by reverse transcriptase activity, viral infectivity, intracellular gs antigen assay and electron microscopic observation all consistently substantiated the fact that the interferon sensitive step preceeds the temperature defect.

Preliminary data also suggested that interferon treatment may result in the formation of defective viral particles. Wild type MLV grown at the non-permissive temperature in the presence of interferon released large numbers of virus particles detected by enzyme assay that were not detected in infectivity assays. Since published data on these temperature sensitive mutants had not taken into account the effect of elevated temperature on the stability of viral enzyme activity or infectivity, experiments are being performed to clarify these effects. Once the kinetics of both virus production and degradation are established at both permissive and non-permissive temperatures, the effect of interferon can more accurately be evaluated. Electron micrographic observations are being made to correlate with data concerning the biologic activity of these particles. A detailed biochemical analysis of the viral RNA and proteins will be used to support the definition of the physical nature of this "defectiveness".

2. Structural Analysis and Transcription of Adeno-Associated Virus DNA: Adeno-associated viruses (AAV) are defective parvoviruses. Because of the small size of their DNA genomes and unique mode of segregation of complementary DNA strands to separate virions, the AAV offer a unique situation amenable to biochemical analysis. AAV synthesizes only a single mRNA species (for its coat protein). Thus the AAV genome seems to consist of a single unique gene enclosed by DNA regions containing reiterated sequences. We are studying AAV as a model system for eukaryotic gene expression. The main objectives of this study are: to analyse the structure of AAV DNA and obtain an accurate transcription map; to study the control of synthesis, post-transcriptional processing and modification and transport and degradation of AAV; and, to study interactions between AAV and its helper adenovirus in both permissive and non-permissive cells. Major findings since the last report are:

a. Cleavage of AAV2 DNA with the restriction endonuclease R.EcoR1 yielded three fragments A, B, and C. Radioactive labeling of the 5'

termini of AAV DNA before cleavage with R.EcoR1 showed that A and B were terminal fragments and C was internal. Separation of the complementary strands of fragments A and B showed that A contained the 5' terminus of the minus strand and the 3' terminus of the plus strand, and conversely for fragment B. The physical map of the AAV R.EcoR1 fragments can thus be unambiguously determined and is drawn with B at the left-hand and A at the right-hand end. On this map, transcription of stable AAV mRNA from the minus strand proceeds from left to right, beginning in fragment B and terminating in fragment A. These experiments enable preparative separation of all four single-strand termini of AAV DNA and provide a basis for orientation of fragment maps derived by cleavage with other restriction enzymes.

b. After HaeIII digestion of duplex DNA terminally labeled with ^{32}P using polynucleotide kinase, the majority of fragments containing a 5' ^{32}P label were about 40 nucleotides in length, and fragments of similar size were generated from each end. This suggests that the Hae site closest to the end is within the terminal repetition. Two more slowly-migrating cleavage products also bore 5' ^{32}P end-label. These three terminally labeled species were also generated from single-stranded AAV DNA by digestion with HaeIII, and evidence was obtained that one may have a nonlinear ("rabbit-ear") structure. The predominant 5' terminal base was identified as thymine for both the plus and minus strands of AAV. Single-stranded AAV molecules could not be efficiently covalently circularized by incubation with polynucleotide ligase or ligase plus T4 DNA polymerase.

c. The synthesis of AAV2 RNA in KB3 cells coinfected with adenovirus as a helper was studied. A discrete 20S AAV RNA species was present in the nucleus and polysomes and a second, heterogenous population of smaller AAV RNA molecules (4S to 18S) was present only in the nucleus and nonpolysomal regions of the cytoplasm. Most of the 20S AAV RNA contained a poly A sequence of 200 nucleutodes in length whereas the heterogenous 4S to 18S AAV RNA contained little or no polyA. Both the poly A(+) and the poly A(-) as well as RNA isolated from the cell nucleus, cytoplasm or polysomes contained the same set of stable AAV RNA sequences complementary to 70 to 75% of the AAV DNA minus strand. These results indicate that a single polyadenylated AAV mRNA species is synthesized and that this represents most, if not all, of the entire portion of the AAV genome that is stably transcribed.

d. RNA-DNA hybridization experiments using various restriction fragments of AAV DNA were performed to obtain a more accurate map of the portion of the AAV genome represented in stable RNA. The data obtained with several sets of restriction fragments annealed to either whole cell RNA or poly A-containing RNA were internally consistent. The AAV RNA annealed with a continuous region of the AAV DNA beginning at 0.18 map units (18%) from the left end of the molecule and ending at 0.88 map units. In addition, the restriction endonuclease BamH-1 was found to make one specific cleavage in AAV2 DNA at 0.22 map units which is 0.04 map units (i.e. 160 nucleotides) to the right ("down stream") of the point corresponding to the 5' end of the viral mRNA.

3. Studies on the biochemical changes in the plasma membrane of interferon treated cells: Studies on the mechanism of action of interferon have been confused by the number of suggestions which appear in the virology literature. These include inhibition of virus uncoating, of virus-directed transcription, of virus-directed translation, or of virus assembly. Recent studies in our laboratory have suggested that a common factor in all interferon induced changes may be an alternation in the plasma membrane of interferon-treated cells. These findings are discussed below.

a. Preliminary studies on the isolated plasma membrane of mouse AKR, C^+ cells showed that there are at least two fractions of membrane separated by equilibrium centrifugation on the discontinuous sucrose density gradient. In membrane preparations of control AKR, C^+ cells, the low density component constitutes about 75% (A_{280}) of the two fractions. However, if the cells were treated with interferon for 16-24 hr. prior to membrane preparation, the distribution of the two density components is reversed. Similar results have also been observed in AKR, C^- cells which do not normally produce virus. The increase in the proportion of the higher density plasma membrane component would therefore seem to be a cellular response to the interferon treatment and the membrane may be the site of interferon action.

b. Additional studies employing the freeze fracture technique to define morpologic alterations in membranes indicate that interferon treated AKR cells have a significant increase in the number of intra-membraneous granules.

c. In Project 4 (below) experiments studying the interactions of interferon, TSH, and cholera toxin are discussed. One aspect of this work has special relevance to the effect of interferon on plasma membranes. When L cells are treated with interferon, there is a complex effect on the subsequent ability of these membranes to bind cholera toxin later. Instead of a simple inhibition of toxin binding, low concentrations of interferon actually cause a marked increase; however, higher concentrations regularly decrease toxin binding and the decrease is directly proportional to the interferon concentration. This complex relationship suggests that interferon causes an alteration in plasma membranes which in turn changes their capacity to bind the toxin. This seems more likely than a direct competition for a common binding site between toxin and interferon since this should never cause an increase in toxin binding.

4. Studies on interferon binding site: The biological activity of interferon is inhibited by cholera toxin (10^{-9} M) or TSH (10^{-5} M). In the case of the cholera toxin, addition of interferon after the toxin is effective in inhibiting the development of antiviral activity. With TSH, however, simultaneous addition is necessary. This is probably related to the tight binding of the toxin.

In addition current work suggests that interferon treatment inhibits the subsequent binding of cholera toxin or TSH to their respective membrane receptors. In the case of the cholera toxin, however, the

effect is quite complex (see section 3). In general these results suggest that interferon either alters the membrane so that it fails to bind toxin or TSH efficiently or that interferon competes with them for specific binding sites. Because of the complex effect of interferon on toxin binding, however, the first explanation is the more likely.

Studies of this nature may lead to the development of simple biochemical assays for interferon. They also suggest methods by which interferon's species specificity might be studied. So far, for instance, interferon has been found to inhibit the binding of TSH or of cholera toxin to the membranes of cells in which the interferon employed has no known biological activity. This preliminary result would suggest that interferon's species specificity is not due to its binding but to a later step in its mechanism of action.

5. Interferon and chronic virus infections: A carrier culture was initiated by pretreatment of L_y cells with mouse interferon followed by infection at multiplicities of VSV of ≥ 30. In the chronic infection virus titers range from 10^3-10^5 plaque forming units (PFU)/ml. The cells appear to be normal except for the presence of a few floating dead cells and a decrease in the number of viable cells. The cultures eventually enter a crisis after 14-24 days and die with viral titers $> 10^6$ PFU/ml. During the course of the chronic infection, however, only one in 100 cells produces infectious VSV, although 10% of the cells form VSV proteins. To understand why we have such a discrepancy between the number of cells producing mature virions and those producing only viral proteins, the cells in the carrier culture will be cloned and the relationship between virus and cells will be studied.

Possible mechanisms of viral persistence are the requirement of defective interfering virus particles of VSV, the development of temperature sensitive mutants of VSV, or interferon production by the chronically infected cells. The chronic infection of VSV in L_y cells will be examined for evidence of T particles (VSV with a defective shortened RNA genome). After labeling the VSV with ^{3}H Uridine, isopycnic density sucrose gradients are being run to purify and concentrate the wild-type and defective VSV. Separation of the normal VSV virions (B particles) and defective T particles will be carried out on velocity sucrose gradients.

The carrier cultures are also being examined for evidence of VSV temperature sensitive mutants. It is possible that we are selecting for VSV virions which do not readily kill the cells and have low infectivity. The cultures will be studied at different temperatures for enhancement or reduction in virus titer and the effect of the virus growth on cell survival. The extracellular viral RNA and protein is being compared to wild-type VSV.

Other experiments are in progress to prove or disprove the induction of interferon by VSV in L_y cells. The culture fluids from this chronic infection show a low level of antiviral activity. The antiviral activity has the biological properties of interferon, but more conclusive evidence can be gathered by isolation and purification of the interferon produced

by these cultures.

At present we are also trying to establish carrier cultures with VSV in cell lines other than L_y. The cell lines will be selected for their ability to produce interferon and their sensitivity to interferon. Also other viruses will be chosen for their capacity to induce interferon and establish chronic infection in L_y cells.

6. Studies on early SV40 antigen formation: Whether a specific viral infection will lead to virus production (lytic infection) or to cell transformation (oncogenesis) depends upon the interactions between virus-specific macromolecules, either carried by the invading virus or coded for by the virus shortly upon infection, with macromolecules specified by the host cell. The study of the synthesis and function of the early SV40 antigens is therefore essential for the understanding of the mechanism of viral infection and oncogenesis. However, these studies have been complicated by the fact that the amount of viral proteins made early post-infection is quantitatively very low, compared to the extent of host macromolecular biosynthesis. To overcome this problem, we have made use of a series of Ad2-SV40 hybrid viruses, each containing varying amounts of the SV40 early genes covalently attached to almost the entire Ad2 genome, which have the ability to turn off the host translational activity upon infection, in order to identify the various SV40-specific early functions.

Using ^{35}S-labelled methionine, we have been able to label virus-specific polypeptides to very high specific activity and to identify on SDS-polyacrylamide gels, SV40-specific polypeptides coded for by the various Ad2-SV40 hybrid viruses. Polypeptides of quite different molecular weights have been detected in cells infected by either $Ad2^+ND_1$ or $Ad2^+ND_2$. Both of these hybrid viruses have previously been shown by immunofluorescent staining to be U-antigen positive and to possess that SV40 early function which apparently allows the growth of human Ad2 in the otherwise nonpermissive simian cells. However, $Ad2^+ND_1$ has been known to be TSTA (tumor specific transplantation antigen) negative while $Ad2^+ND_2$ to be TSTA positive by cytotoxicity test. We have purified each of these [^{35}S]labeled polypeptides and are comparing their respective tryptic peptide fingerprint to determine whether the smaller 28K protein found in $Ad2^+ND_1$-infected cells is a subset of the larger 42K or 56K proteins observed in $Ad2^+ND_2$-infected cells. This will definitively show whether the 28K protein is actually a functional 'deletion mutant' protein of the larger polypeptides found in $Ad2^+ND_2$. We are also trying to determine whether any of these purified polypeptides are immunologically reactive to U^+-antisera and whether any of these polypeptides possesses TSTA activity by cytotoxicity tests. We have been able to localize each of these SV40-specific polypeptides to specific sub-cellular fractions or 'functional sites'. We are now searching for any biochemical change that may accompany the appearance of a specific viral antigen upon virus infection, and with the data obtained from the localization studies, designing cell-free systems to probe the functions of specific antigens.

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Friedman, R. M., Costa, J. C., Ramseur, J. M., Myers, M. W., Jay, F. T., and Chang, E. H. Persistance of a viral genome in interferon-treated cells infected with oncogenic or nononcogenic viruses. J. Infectious Diseases (in press), 1976.

Friedman, R. M., Jay, F. T., Chang, E. H., Myers, M. W., Ramseur, J. M., Mims, S. J., Triche, T. J., and Wong, P. K. Y. Interferon-directed inhibition of chronic murine leukemia virus production in cell cultures. Proceeding of Conference on Antitumor Agents, Fogarty Intl. Center Proceedings (in press), 1976.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 21,005-03 LEP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Histochemistry: principles, methods, and applications

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

PI:	N. Feder	Chief, Section on Biophysical Pathology	LEP:NIAMDD
OTHER:	W. Stewart	Staff Fellow	LEP:NIAMDD

COOPERATING UNITS (If any)

None

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Biophysical Pathology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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

Cytochemical methods have been used to determine the position of substances in tissues and cells. (1) β-Glucuronidase has been applied as a cell marker in tetraparental mice. This approach has been used to study cell movement and enzyme exchange between cells. (2) Two new highly fluorescent tissue-reactive dyes (N-substituted naphthalimides) have been used to mark individual neurons in vivo through recording microelectrodes.

PHS-6040
(12-75)

Project Description:

Beta-glucuronidase as a Cell Marker: Studies have been concluded on chimeric (tetraparental) mice with β-glucuronidase as a cell marker. The chimeric mice were made from pairs of inbred mouse strains whose tissues are known to differ in β-glucuronidase activity. Thus the mosaic tissues of the chimeric mice were composed of two intermingled populations of cells which could be distinguished microscopically in tissue sections stained histochemically for β-glucuronidase. (a) In many of the mosaic tissues there were small clusters of cells (i.e., cells of one staining type -- say high-glucuronidase cells -- surrounded by cells of the contrasting type). Small clusters of cells and solitary cells were found in a wide range of tissues, primarily epithelial tissues. This finding contrasts with several reports by other investigators that mosaic tissues are composed of large clusters -- in the range of 10^4 to 10^8 cells per cluster. The presence of small clusters and solitary cells supports but does not prove the conclusion that cell migration may continue in many tissues throughout their growth. (b) Staining differences between cells of chimeric mice and cells of the same tissues in single-genotype control mice showed some interesting regularities consistent with the hypothesis that there is an exchange of enzyme between cells. This hypothesis receives support from the preliminary finding that some hepatocytes of chimeric mice contain enzyme characteristic of both the high-glucuronidase and low-glucuronidase strains of mice. These observations, together with reports by other investigators that exogenous lysosomal enzymes are taken up by cells in the intact animal and in cell culture, support the view that exchange of glucuronidase (and other enzymes) among cells actually occurs as a normal function in a wide range of cell types.

Marking Neurons for Light and Electron Microscopy: Studies with two highly fluorescent tissue-reactive dyes (N-substituted naphthalimides) have been concluded. The dyes, which are now synthesized on a large scale and in high yield, are analytically pure. (a) These dyes have been used to mark individual neurons *in vivo* through recording microelectrodes. Successful marking of a wide variety of vertebrate and invertebrate neurons has now been carried out in laboratories at the N.I.H. and elsewhere. (b) The occurrence of clusters of coupled neurons has been confirmed by injection of a single neuron and observing spread of the dye to contiguous neurons. (c) By injection of dye into the eye of the chick embryo, it has been possible to demonstrate reverse axonal transport along the optic nerve to the isthmo-optic nucleus. Two further studies are in progress. (d) The naphthalimide dyes are under investigation as labels of γ-globulin and other proteins. The high fluorescence efficiency of the dye and certain other properties make it promising as a label for proteins. (e) Studies on marking of single neurons for electron microscopy with heavy metals are in progress. These studies have until now been unsuccessful.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01AM21006-08LEP

PERIOD COVERED

TITLE OF PROJECT (80 characters or less)

Protein and Proteolytic Enzyme Interrelationships in Normal and Disease States.

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

PI: George G. Glenner, Chief, Section on Molecular Pathology, LEP/NIAMDD

COOPERATING UNITS (If any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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

It is the purpose of this project to study the chemistry and structure of amyloid and amyloid related proteins. The main topics of present interest are:

1) Isolation and chemical characterization of amyloid fibril protein from various types of systemic amyloidosis and localized amyloid deposits.

2) Mechanism of formation of amyloid deposits by sysosomal enzyme digestion of immunoglobulin precursors.

3) Isolation and purification of proteolytic enzymes involved in immunoglobulin degradation.

PHS-6040
(12-75)

Project Description: Z01AM21006-08LEP

Removing Low Molecular Weight Proteins from Urea and Guanidine HCl Solutions. Differential solubility in organic solvent mixtures provides the basis of an alternative method for removing guanidine HCl and urea from protein solutions. Solubilization and removal of these denaturing agents are accomplished by the use of specific combinations of organic solvents of varying dielectric constant, added to dehydrated mixtures of protein and crystalline guanidine HCl and urea. For the proteins tested, recovery varied directly with molecular weight.

Reaction of Amyloid Protein with Lipid. Polypeptide segments, composed of α-helixes with specific surface topography termed amphipathic helixes, have been proposed as the basic lipid associating domains of apolipoproteins from the plasma lipoproteins. A computer search for proteins having sequences which could form amphipathic helixes indicated that amyloid A, a pathologically occurring protein usually associated with "secondary" amyloidosis, also contained amphipathic helixes. In studies reported here amyloid A is shown to spontaneously associate with phospholipid vesicles with the following results: a) the formation of a protein: lipid complex isolated by equilibrium density gradient ultracentrifugation, b) a 100% increase in α-helicity as measured by circular dichroism, c) a 9 nm shift in the fluorescence maximum due to the single tryptophan residue located in the amphipathic region, indicating the tryptophan is moving from a polar to a nonpolar environment and, d) the formation of stacked disc-like protein: lipid complexes as visualized by negative stain electron microscopy. The temperature dependence of the circular dichroid spectrum of the amyloid A: phospholipid complex suggests that the complex is formed by insertion of protein between the fatty acyl chains of the lipid. These findings suggest that the amphipathic helix is an important structural unit in lipid associating proteins and that this unit can be recognized on the basis of its amino acid sequence. In addition, these studies have implications for the origin and function of amyloid A protein.

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1. Linke, R.P., Sipe, J.D., Pollock, P.S. Ignaczak, T.F. and Glenner, G.G.: Isolation of a Low-Molecular-Weight Serum Component Antigenically Related to an Amyloid Fibril Protein of Unknown Origin. Proc. Natl. Acad. Sci. USA. 72: 1473-1476, 1975.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01AM21007-03LEP

PERIOD COVERED

TITLE OF PROJECT (80 characters or less)

Immunochemical Analysis of Proteins Associated with Amyloidosis.

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

PI: Jean D. Sipe, Staff Fellow, LEP/NIAMDD

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	1	3

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

It is the purpose of this project to study the chemistry and antigenic pecularities of amyloid proteins and their progenitors. The main topics of present interest are:

1) Immunochemical characterization of amyloid proteins from various types of systemic amyloidosis and localized amyloid deposits.

2) Immunochemical characterization of abnormal circulating proteins in various disease ststes, e.g. amyloidosis.

3) Amino acid analysis and sequences of pathologic proteins.

4) Application of proteolytic enzymes in protein chemistry.

PHS-6040
(12-75)

Project Description: Z01AM21007-03LEP

Human Secondary Amyloidosis. Amyloid of unknown origin (AUO) is the designation for a class of amyloid fibrils deposited in a form of systemic amyloidosis which occurs in man and other classes of animals and which is distinct from human amyloidosis of immunoglobulin origin. The major protein contained in these fibrils is called AA and its amino acid sequence does not correspond to that of any known protein. Since the detection, in serum, of a component called SAA with antibodies raised to denatured AA containing A fibrils, there has been considerable interest in the isolation of SAA both for biochemical studies of its role as a possible precursor of the AA protein deposited in tissues, and for use as an immunogen for immunochemical studies to identify the cell of origin of SAA. Work carried out in several laboratories, including this one, has shown that SAA has the properties of an acute phase reactant in man, mink, and mouse, and it has been reported that murine SAA has an immunosuppressive effect. To date, the only form of SAA which has been purified is a polypeptide chain of 12,500 m.w., SAAL, which has been isolated from serum by gel filtration in guanidine HCl or 10% formic acid, or electrophoresis on SDS polyacrylamide gels.

The native structure of SAA is unknown; it is possible that SAAL is complexed through noncovalent bonds to a high m.w. polypeptide which does not cross-react with anti-AA antibodies, or that SAA is a polymer of SAAL polypeptide chains arranged in an unknown fashion, perhaps containing additional micromolecular material such as peptides or lipids. One experimental approach to determine whether there was a high molecular weight polypeptide carrier for the SAAL polypeptide, was to radiolabel the SAA fraction from whole serum with iodine 125 and to then mix it with unlabeled, whole, SAA-containing serum and carry out immunoprecipitation with anti-AA antibodies using a double antibody technique. The immunoprecipitated ^{125}I-SAA was analyzed by SDS urea-polyacrylamide gel electrophoresis and only a fraction of the precipitated radioactivity was observed to migrate with a m.w. of 12,500. Multiple radiolabeled species and molecular weights intermediate to SAA and SAAL were also detected and appear to represent incompletely dissociated SAA, suggesting that SAA is an aggregate of several SAAL chains.

The isolation of SAA was greatly hampered by the lack of quantitative assay. During the past year, a solid phase radioimmunoassay for AA, which employs purified antibodies to AAE (the major fraction purified from AA by isoelectric focusing) adsorbed onto the wells of flexible microtitration plates, was applied to the purification of SAA from serum. In addition to facilitating the isolation procedure, the use of the RIA for monitoring the fractionation of SAA-containing serum has revealed information about the conformation and flexibility of SAA.

Several properties of SAA suggest that in its native conformation the antigenic determinants which cross-react with antibodies raised to AA are relatively inaccessible or buried. The native molecular weight of SAA appears to be approximately 160,000 as determined by gel filtration of fresh serum in PBS. SAA appears to be labile in that it both aggregates and dissociates upon storage or upon usually non dissociating manipulations. There is an increase in immunoreactivity of SAA-containing serum upon storage and

experimental manipulation which does not normally dissociate noncovalent bonds, as well as upon isolation of SAAL by gel filtration under dissociating condition. This increase, relative to whole serum, is two fold for the isolation of SAA by gel filtration, four fold for the isolation of guanidine HCl denatured SAAL, and almost 150-fold for the isolation of SAAL in the presence of formic acid. RIA determination of SAA in whole serum can reach levels of 5 µg/ml of AA cross-reactivity, yet sera with cross-reactivity as low as 50 ng/ml are strongly reactive in double immunodiffusion. It appears that unfolding of cross-reacting determinants occurs during the process of double immunodiffusion.

Qualitative immunodiffusion assays were compared with RIA for SAA. Except in sera from individuals with multiple myeloma, higher levels of SAA correlated with positive immunodiffusion assays. Ninety-three percent of the pathological sera with SAA levels greater than 100 ng/ml had positive immunodiffusion tests. For approximately 150 normal sera a median value of 8 ng/ml SAA was observed with no age related increase in subjects ranging in age from 16-70 years. SAA levels tended to be high in certain categories of neoplastic, inflammatory and infectious diseases, as well as in secondary amyloidosis. Less elevation was observed in primary amyloidosis. The results are consistent with many recent observations that SAA levels fluctuate like acute phase reactants, are in essential agreement with a previously reported study by Rosenthal and Franklin which employed an RIA different in principle from the one used in this laboratory. The RIA for SAA has no prognostic diagnostic significance for secondary amyloidosis at the present time.

Murine Model for Human Secondary Amyloidosis. Murine amyloidosis, which can be induced by a wide variety of antigens including endotoxin, mycobacterium in complete Freunds adjuvant, and casein; and which occurs "spontaneously" in some strains of mice under crowded conditions; has been found to be a suitable experimental model for human secondary amyloidosis. Previous studies from this section described similarities of morphology of the amyloid fibrils (which consist of protein AA), and in organ distribution, between murine amyloidosis and human secondary amyloidosis. Our present studies show that there is a murine serum protein (SAA) which is very similar to the putative serum precursor of human secondary amyloid fibrils in that: 1) Murine SAA exists in the native state as a 160,000 m.w. species in a conformation such that the determinants which cross-react with AA are inaccessible. Prior denaturation of serum in formic acid is required for maximal detection of SAA by radioimmunoassay. 2) Murine SAA is dissociated to a species of 12,500 m.w. (called SAAL) upon chromatography on Sephadex G-100 in 10% formic acid and upon polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

The serum protein SAA, which is thought to be the precursor of the fibril protein deposited in human secondary amyloidosis and in most of the amyloidoses observed in experimental animals, has recently been characterized in collaborative studies involving this section, as an acute phase protein in the mouse.

Levels of murine SAA are observed to rise within 5 hours after injection of an amyloid inducing agent such as lipopolysaccharide (LPS), casein Hammersten or azocasein, to increase several hundred fold by 24 hours and to return

to basal levels by 48 hours. When various types of LPS preparations are employed to induce SAA, there is a close genetic correlation between SAA production and the physiological responses observed by other workers (in vivo: immune response, adjuvant effect, lethality; in vitro: mitogenicity) to these polyclonal B cell mitogens.

Preliminary experiments show that at the height of the SAA response immunocross-reactivity is observed in the liver and kidney to a much greater extent than the spleen which is the first site of fibril deposition approximately 7 days later.

Primary Structure of Amyloid Proteins. To date, two classes of systemic amyloid proteins have been chemically characterized: amyloid of immunoglobulin origin and amyloid of unknown origin. Costa and coworkers have identified a third class of protein deposited systemically in a heredofamilial form of amyloidosis. Our immunochemical studies have shown that the major protein solubilized from these deposits cross-reacts with antibodies raised to a preparation of normal human prealbumin, the chemical purity of which has been established by amino acid sequence determination of the first thirty residues from the amino terminus. We have established in collaboration with Waxdal and coworkers a microsequencing methodology employing the Beckman spinning cup sequences, ^{35}S-PITC,and thin layer chromatography with autoradiographic determination, which can identify at least thirty residues using approximately 50 nmoles of protein. Preliminary sequence data show both sequence similarities and some structural differences between the familial amyloid protein and the normal prealbumin.

Future plans for this project include structural studies on human and murine serum proteins SAA, which are antigenically related to protein AA isolated from amyloid deposits of unknown origin, and which are acute phase reactants. The amino and carboxyl terminal heterogeneities of AA (m.w. 5300-9000) suggest that it results from proteolytic digestion of a larger precursor. This precursor is thought to be SAAL (12,500 m.w.) which can be obtained from SAA (160,000 m.w.) by gel filtration under conditions which dissociated noncovalent bonds. AA is of similar sequence between different human and murine individuals, but it is not known whether the addition unsequenced portions of the polypeptide chain vary. The objectives of this study include isolation and comparison of those peptides from SAAL which are not contained in AA.

Immobilized Pyrrolidone Carboxylate Peptidase (PCA'ase). Previous studies had shown that PCA'ase coupled to CNBr activated agarose was active toward peptides but not polypeptides. We have recently found that the immobilized enzyme also is not reusable, in that it loses approximately 80% of its activity with each cycle of usage. One possible explanation is that the enzyme is being released from the matrix at a much higher rate than normal. There are no current plans to continue this project, because the enzyme in solution appears to be comparable or better to the immobilized enzyme.

References:

1. Linke, R.P., Sipe, J.D., Pollock, P.S., Ignaczak, T.F. and Glenner, G.G.: Isolation of a low-molecular-weight serum component antigenically related

to an amyloid fibril protein of unknown origin. Proc. Natl. Acad. Sci. USA. 72: 1473-1476, April 1975.

2. Sipe, J.D., Ignaczak, T.F., Pollock, P.S., and Glenner, G.G.: Amyloid fibril protein AA: Purification and properties of the antigenically related serum component as determined by solid phase radioimmunoassay. J. Immunol. 116: 1151-1156, April 1976.

3. McAdam, K.P.W.J., and Sipe, J.D.: Serum percursor of murine amyloid protein: An acute phase reactant in response to polyclonal B cell mitogens. Federation Proceedings, (in press), Abstract.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-21008-11 LEP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cytogenetics

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

PI:	J.-H. Tjio	Chief, Section on Cytogenetics	LEP NIAMDD
Other:	B. J. White	Acting Chief, Section on Cytogenetics	LEP NIAMDD

COOPERATING UNITS (if any)

D. Germain	University Claude-Bernard, Lyon, France	
G. Brecher	Univ. Calif. Medical Center, San Francisco	
W. W. Smith	LPHY	NCI
A. Steinberg	A&R	NIAMDD

LAB/BRANCH

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NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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

1) Development of new chromosomal differential staining techniques and continued improvement of established methods are basic to all of the projects conducted in the laboratory. During the past year, two more precise methods for studying chromosome structure and function were introduced: sister chromatid exchange, a sensitive means of visualizing chromosomal damage and repair, and analysis of chromosomal replication sequences by differential staining, a technique which replaces autoradiography. These improved methods were then utilized for all major studies in progress.

2) Human cytogenetic studies of patients with congenital and developmental disorders as well as studies of mutagen-induced chromosomal abnormalities are continuing. In addition, differential staining methods are now being used to study patients exposed to benzene and related polycylic hydrocarbons.

3) Murine cytogenetic studies include evaluation of mitotic and meiotic chromosomes of Robertsonian translocation carriers and karyotypic evaluation of cells in hematopoietic tissue regeneration experiments. These are part of long term projects utilizing the mouse as a model for human disorders.

PHS-6040
(12-75)

Dr. Tjio is currently on sabbatical in the laboratory of Dr. Daniel Germain at the University Claude-Bernard in Lyon, France.

Project Description:

Cytogenetics of Hematopoetic Disorders.

These studies are being carried out in the laboratory of Dr. D. Germain (University Claude-Bernard, Lyon, France). The newly-developed differential staining methods are being employed to analyze abnormal hematopoetic cells found in patients with hemopathies such as Fanconi's anemia and in patients exposed to leukemogenic substances such as benzene and related polycylic hydrocarbons. With various banding techniques, they are attempting to detect specific chromosome anomalies in direct marrow preparations as well as in cultures established from hemopoetic tissues. Similar studies are being conducted with mice to develop an animal model for the chromosome alterations induced by polycylic hydrocarbons which will correlate with the human studies.

Chromosome Identification and Studies of Chromosomal Abnormalities Uaing Differential Staining Techniques.

The Giemsa-, quinacrine- and centromeric-banding methods are being employed to identify chromosomes and to detect variations in both the human and laboratory mouse karyotypes. Some form of differential staining is almost always utilized in our initial analysis, with further investigations made using more complex banding methods if indicated by our initial findings. Since some of the differential staining techniques currently being used in France are different than those commonly used in our laboratory (reverse-banding and acridine fluorescence), Dr. Tjio is becoming familiar with these procedures and will be utilizing them here upon his return.

Our laboratory continues to study sister chromatid exchange (S.C.E.) in chromosomes of somatic cells. This method involves in vitro replication of cells in the presence of 5-bromodeoxyuridine (BUdR), treatment with the benzimidazole derivative Hoechst 33258, and exposure to ultra-violet light. After staining with Giemsa, exchanges between the two chromatids of a chromosome can be visualized. We are in the process of establishing the normal S.C.E. rate in our laboratory. During the past year we have used this method in studying several mouse strains and for specific patient studies; the S.C.E. rate being a more sensitive indicator of chromosome damage than visible morphological alterations. We will also be able to apply this technique to studies of patients with possible chromosome breakage syndromes and those exposed to potential mutagens.

Replication sequences can now be explored in our laboratory with differential staining. The addition of thymidine (TdR) to cells growing in a medium containing 5-bromodeoxyuridine (BUdR) at the end of the first DNA replication cycle (S phase) results in the incorporation of TdR into late replicating chromosomal regions. These sites can be visualized by staining metaphase chromosomes with the same Hoechst-Giemsa procedure that is utilized for the S.C.E. technique. The early replicating sites of BUdR incorporation stain less intensely with Hoechst-Giemsa than the late replicating regions that have incorporated TdR, making it possible to determine the time during the S period that specific chromosomal bands and segments replicate. The reverse of this method involves the addition of BUdR during the last few hours of the S phase. Late replicating regions can be visualized with this

variation of the technique as light staining regions. The banding patterns seen following both methods of treatment agree with sequences of replication demonstrated by standard autoradiographic techniques, but are more accurate and precise. These techniques are now being used by us to study patients with abnormal sex chromosomes.

Meiotic Chromosomes of the Female Mouse.

In the past, meiotic nondisjunction could only be studied directly in testicular preparations from the male mouse, and in the female could only be indirectly determined by studying chromosomally abnormal embryos. Using a method developed by Dr. I. Uchida (McMaster University, Hamilton, Canada), we can now study meiotic chromosomes from mouse oocytes. The technique involves freeing the oocytes from the ovaries and isolating them under a dissecting microscope, a short-term incubation period to obtain meiotic metaphase I or II cells, and hypotonic treatment followed by fixation. We will be employing this technique to more accurately determine the rate of meiotic nondisjunction in female mice heterozygous for three Robertsonian translocation chromosomes. In turn this will help us to determine if maternal factors, other than nondisjunction rate in the gonadal cells, play a role in the production of trisomic embryos.

Pattern of Recovery of Hemopoiesis in the Mouse After Irradiation and Mechanical Depopulation.

Using the AKR-T1A1d marker chromosome system, we have established that donor hemopoietic stem cells fail to survive and proliferate in ectopic sites in normal syngeneic hosts but do so in irradiated hosts. In collaboration with Dr. G. Brecher (University of California Medical Center, San Francisco) and Dr. W. W. Smith (NCI), these studies on the hematopoietic differentiative properties of murine spleen implanted in the omenta of irradiated and nonirradiated hosts have been published.

Work on recovery of marrow from mechanically depleted femurs is now being published. We have demonstrated that hemopoietic repopulation in the depleted femurs is invariably of the same composition as the pre-existing mix of donor and host cells in that limb; e.g., in chimeras produced by T1A1d marrow transfused into AKR mice after high doses of radiation, repopulation is entirely from donor (T1A1d) type. After local shielding, when a mixture of host and donor hemopoietic cells is established in the shielded limb by post-radiation marrow transfusion, repopulation after mechanical depletion is also of the mixed type. Our karyotypic determination of cultured stroma of the repopulating marrow showed that the stroma is of host origin. Presumably, bone is formed from these stromal cells. These observations indicate that there does not exist a common stem cell precursor of bone and marrow cells in repopulation after mechanical depletion, since the stromal cells are solely of host type even when hemopoietic marrow cells are all donor or mixed. Experiments were also conducted to study marrow regeneration in ectopic transplants. Observation of normal hemopoiesis in transplants of marrow fragments or femurs in the absence of bone formation, and the lack of correlation between bone formation and normal recovery after various types of marrow depletion make a causal relationship between bone formation and hemopoietic regeneration highly unlikely. Radiation can significantly alter environmental conditions so that new bone formation is largely suppressed

without suppressing hemopoiesis. In the mechanically depleted femurs, new bone formation appears to occur in close conjunction to areas of local trauma or organization of a clot in the medullary cavity. Regardless of the conditions which suppress or give rise to osteogenesis, it is now evident that with an intact stroma, ectopically grafted marrow is able to grow and proliferate without concomitant osteogenesis.

Again using the AKR-T1A1d system, we plan to further investigate why grafts of isologous donor marrow into normal hosts are successful only when recipients have been subjected to doses of ionizing radiation or radiomimetic drugs. We hope to explore whether host stem cells have a competitive advantage over injected donor cells or whether some proliferative stimulus is needed to activate the donor cells.

Clinical Cytogenetic Studies of Human Congenital and Developmental Disorders.

Patients referred to our laboratory for karyotypic diagnosis are those with various unusual genetic disorders, abnormalities in gonadal development or function, and selected cases of infertility in the male. In these types of cases, we are looking for minor variants or abnormalities as well as gross morphological changes. With routine karyotyping many of these anomalies could be overlooked or misinterpreted; therefore, all preparations from such patient referrals are Giemsa- and quinacrine-banded. Additional procedures such as centromeric-banding, sister chromatid exchange, and/or late replication studies are used if a chromosome abnormality is found or suspected. With the addition of new and improvement of our older, more standard banding procedures, we are now better prepared to detect karyotypic variations and more capable of correctly interpreting them.

Murine Immunogenetic Studies.

Experiments are currently being performed in collaboration with Dr. A. Steinberg (NIAMDD) to determine the effect of the NZB genetic background on the immunological status of NZB/DBA F_1 hybrid mice. The NZB strain, characterized by immunological abnormalities of the autoimmune type, has naturally occurring thymocyte antibodies. We have detected no such antibodies in the NZB/DBA F_1 males, and have determined that castration alters this failure to respond. However, F_1 females normally produce these thymocyte antibodies, and removal of the ovaries does not alter this response. Cytogenetic studies, including sister chromatid exchange, are currently being performed on these mice. We hope to correlate the immunological and cytogenetic findings.

Publications:

Haley, J.E., Tjio, J.H., Smith, W.W., and Brecher, G.: Hematopoietic differentiative properties of murine spleen implanted in the omenta of irradiated and nonirradiated hosts. Exp. Hemat. 3: 187-196, 1975.

Brecher, G., Tjio, J.H., Smith, W.W., and Haley, J.E.: Marrow regeneration after mechanical depletion. Blood, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-21009-06 LEP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cytogenetic Studies

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

PI:	B. J. White	Acting Chief, Section on Cytogenetics	LEP NIAMDD
Other:	J.-H. Tjio	Chief, Section on Cytogenetics	LEP NIAMDD

COOPERATING UNITS (if any)			
	R. Johnsonbaugh	Dept. Pediatrics	NNMC, Bethesda
	W. Rowe	LVD	NIAID
	W. Caveness	LEN	NINCDS
	D. Symmes	BB	NICHD

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Cytogenetics

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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

1) Cytogenetic and reproductive studies of murine Robertsonian translocation carriers. Analysis of adult and fetal somatic and gonadal tissues using chromosomal banding techniques in order to study meiotic nondisjunction and characteristics of aneuploid progeny from translocation heterozygotes.

2) Cytogenetic and dermatoglyphic analysis of patients with congenital defects, recognized dysmorphic syndromes, abnormal gonadal development and function, and other genetic disorders. Techniques used for cytogenetic studies include Giemsa-, quinacrine-, and centromeric-banding, as well as sister chromatid exchange and fluorescent-Giemsa analysis of chromosomal replication sequences. Cytogenetic, dermatoglyphic, and other clinical studies are correlated.

3) Karyotypic evaluation of potentially abnormal tissues. These studies utilize a variety of chromosomal banding methods for analysis of tissues from laboratory animals (primate and murine) suspected of having chromosomal abnormalities. Relationship of cytogenetic findings to genetic background, development of tumors or malignant transformation, and reproductive fitness is emphasized.

PHS-6040
(12-75)

Project Description:

1) Cytogenetic and reproductive studies of murine Robertsonian translocation carriers.

Since 1967, we have studied a series of Robertsonian translocations in the laboratory mouse. Three basic strains established by us have been the most extensively studied: Rb(5.19), Rb(6.15)/Rb(5.19), and Rb(6.15)/Rb(5.19)/Rb(8.17). Rb(5.19) is currently in generation 36 of inbreeding; it has recently been utilized to produce allophenic mice for cleft palate studies by investigatora outside of NIH. Their results is confirmed by others, suggest that the Rb(5.19) strain may become a major one used for cleft palate induction experiments. We are continuing our characterization of the most recently established triple tranalocation stock, Rb(6.15)/Rb(5.19)/Rb(8.17). Cytological studies of male triple homozygotes show that their rate of meiotic nondisjunction is less than 1.0%. Male triple heterozygotes cytologically have 25% aneuploid spermatocytes. During the past year we have found that our meiotic chromosome analysis of males correlates with the actual frequency of aneuploid fetuses detected in female heterozygotes at 10 days' gestation. At that stage 12% of embryos are trisomic, half for chromosome 19, and half for the other chromosomes involved in the three translocations (numbers 5, 6, 8, 15, 17). We are still conducting these experiments. In the future, we plan to compare the frequency of trisomic embryos in male and female triple heterozygotes and correlate this with results of karyotypic and morphological studies of embryos. We are particularly interested in determining if different genetic backgrounds influence the types of malformations found in aneuploid embryos and fetuses. Since we maintain, in addition to the three strains mentioned, three other lines homozygous for single isolated translocations (Rb(6.15), Rb(8.17), and Rb(9.19)), we can test these effects in mice with trisomy for the translocation chromosomes specific for each strain.

2) Cytogenetic and dermatoglyphic studies of patients.

These studies of patients with congenital defects, recognized dysmorphic syndromes, abnormal gonadal development and function, and other genetic disorders have been continued during the past year. In order to do the chromosome studies, a variety of differential staining techniques have been employed to do detailed studies of chromosome structure; cases were selected for research interest so that structure of specific types of chromosome rearrangements and abnormalities could be studied. The banding techniques carried out in our laboratory for the past several years that give detailed analysis of the structure of chromosome arms and centromeres include Giemsa-, quinacrine-, and centromeric-banding (G-, Q-, and C-banding). The first two techniques are used in all cases and the latter when rearrangements involving the region of the centromere are detected. We recently added two new techniques to those already in use: Sister Chromatid Exchange (S.C.E.) is a phenomenon noted in Giemsa-stained metaphses when cells are cultured in the presence of bromodeoxyuridine (BUdR), stained with the benzimidazole derivative Hoechst 33258, and treated with ultra-violet light. Metaphases from normal individuals have only a small number of S.C.E.'s per cell following this treatment. In Bloom's syndrome, an autosomal recessive disease characterized by high chromosome breakage rates, the S.C.E. rate has been found by others to be tremendously increased. Investigations by others also indicate that the

exchange rate is increased by alkylating agents in a dose-related fashion, and that S.C.E. is a more sensitive indicator of chromosome damage than traditional morphological methods. At present, we are currently determining the normal control rate of exchanges in human lymphocyte cultures and have studied several patients with this technique. The method is also being used in our lab for studies of several mouse strains and will continue to be utilized for studies of patients with suspected exposure to mutagens or possible chromosome breakage syndromes. Replication sequences with differential staining: The addition of thymidine (TdR) to cells growing in a medium containing 5-bromodeoxyuridine (BUdR) at the end of the first DNA replication cycle (S phase) results in the incorporation of TdR into late replicating chromosomal regions. These sites can be visualized by staining metaphase chromosomes with the same 33258 Hoechst-Giemsa procedure that is utilized for the S.C.E. technique. The early replicating sites of BUdR incorporation stain less intensely with Hoechst-Giemsa than the late replicating regions that have incorporated TdR, making it possible to determine the time during the S period that specific chromosomal bands and segments replicate. The reverse of this method involves the addition of BUdR during the last few hours of the S phase. Late replicating regions can be visualized with this variation of the technique as light staining regions. The banding patterns seen following both methods of treatment agree with sequences of replication demonstrated by standard autoradiographic techniques, but are more accurate and precise. These techniques are now being used by us to study patients with abnormal sex chromosomes (isochromosome X and non-fluorescent Y), and are also necessary when our other banding methods (C-, G-, and Q-banding) cannot permit us to identify abnormal chromosomes.

Short stature: We have continued our study of frequency of sex chromosome defects in pre-pubertal and teen-aged girls in collaboration with Dr. R. Johnsonbaugh (NNMC, Pediatric Endocrinology). During the first year, 4 of 13 cases had abnormal karyotypes (30.8%). This past year, 4 of 14 cases were abnormal (28.6%), supporting our initial impression that the population of short girls with parents of average or above average stature has a very high frequency of sex chromosome defects such as 45,XO, 45,XO/46,XX, or 45,XO/46,XY. This survey will be continued during the coming year, and several of the more unusual patients will be described in case reports. As an outgrowth of the short stature study, Dr. Johnsonbaugh has initiated a study with our laboratory and the NNMC Pediatric Cardiology group to determine the frequency of sex chromosome defects in females with left-sided congenital heart disease. All of six cases completed so far have had normal karyotypes.

Congenital defects, dysmorphic syndromes, and genetic diseases: During the year, a total of 63 cases were studied; 23 from NNMC and 40 from various institutes at NIH. Twelve cases of Kallman's syndrome (hypogonadotropic hypogonadism) from NIAMDD were of special interest. The banded karyotypes of five females and seven males with the syndrome were normal, a finding consistent with other evidence that it is an autosomal recessive disorder rather than a syndrome associated with a visible chromosome defect. Dermatoglyphic analysis is also being done by our lab on this group of patients. The remaining 28 consultations from NIH were predominantly syndromes of unknown etiology and atypical cases of gonadal dysgenesis, all requiring banding rather than routine karyotyping. Of this latter group, three cases of pericentric inversion for chromosome number 9, four cases of Klinefelter's

syndrome, and five cases of low grade mosaicism for sex chromosome aneuploidy were detected. Of all 63 cases studied this past year, 20 were abnormal (31.8%), reflecting our selection of only those cases of research interest.

3) Karyotypic evaluation of potentially abnormal tissues.

Murine tumors: During the past year, we have collaborated with Dr. W. Rowe (NIAID) in chromosomal analysis of AKR mouse thymomas. Others have reported preferential trisomy for chromosome 15 in direct preparations of these tumor tissues and have suggested that there is a genetic relationship between loci on this chromosome and appearance of tumor in the thymus. At present, we are attempting to confirm these results and are analyzing with banding techniques the karyotypes of thymic tumors of AKR hybrids carrying specific genetic loci known to be related to tumorigenesis. During five experiments, we have analyzed direct preparations of thymic tissue as well as peripheral lymphomatous tissues. Since direct preparations of tumors are technically difficult to band, we utilized short-term cultures and transplanted tissues in some instances. So far, we have not detected preferential involvement of any single chromosome and have determined that the tumor tissues fall into two groups: those with frequent nonspecific chromosomal abnormalities and those which are predominantly karyotypically normal. Not enough experiments have been completed to determine if the karyotypic findings are related to specific genetic loci, and more animals will be studied during the coming year. Also, the technique of direct preparation of tumor tissue needs to be improved further in order to give unequivocal results.

Primate chromosomes: We are continuing to collaborate with Dr. W. Caveness (NINDS) in chromosome studies of tissue culture from irradiated Rhesus brain. Our co-investigators are attempting to clarify the mechanism of endothelial cell proliferation seen after radiation-induced brain damage with biochemical, viral, and pathological techniques. At present, we are attempting to improve growth of brain tissue by using selective media, since poor growth of damaged as well as normal tissues has limited most of the past experiments. We are also continuing to collaborate with Dr. D. Symmes (NICHD) in karyotypic studies of a series of chromosomal inversions in squirrel monkeys. This is a very long term study attempting to correlate behavioral and genetic factors, and may provide a primate model for inversion effects in humans.

LABORATORY OF CHEMICAL BIOLOGY, NIAMDD
ANNUAL REPORT: JULY 1, 1975 - JUNE 30, 1976

The overall program of the Laboratory of Chemical Biology continues to be concerned with the chemistry, function and three-dimensional conformation of macromolecules. Most of the work relates to proteins, although some studies have been carried out during the past year on nucleic acids and nucleoproteins. A wide variety of proteins has been investigated and will be considered individually below. Of the problems with a more or less direct bearing on human disease, the most active relate to the examination of hemoglobin S and its behavior in cells, and on human interferon, which shows promise as a potential preventative or palliative agent in a variety of viral diseases.

I. Further development of methodology for research on macromolecules

A. Isolation and purification of transfer ribonucleic acid (tRNase)

The ethidium cation of the salt ethidium bromide has been previously shown by others to be a sequence specific nucleic acid intercalating agent. We have covalently linked the ethidium cation to agarose gel beads through a spacer arm to produce an affinity chromatography system for nucleic acid. The interaction of the gel bound ethidium ion into nucleic acid double helices has been demonstrated by a variety of biophysical techniques including fluorescence. Preliminary experiments indicate that crude transfer ribonucleic acid can be fractionated to some degree on this column using elution with a linear sodium chloride gradient. (Thomas and Schechter)

B. The action of Freud's adjuvant on protein conformation

Use of complete Freund's adjuvant for production of antibodies to study protein conformation is valid only if emulsification in adjuvant does not denature protein antigens. Using electron paramagnetic resonance to observe directly the protein in situ in the opaque emulsions, we demonstrate that hemoglobin is not denatured by emulsification or storage in adjuvant for 24 hours at 4°C. The results also rule out one proposed mechanism of adjuvant action, via alteration of protein structure, at least in this case. (Berzofsky and Schechter) (Cooperating Unit: NIAMDD:LCP)

C. Quantitative affinity chromatography

Affinity chromatography of staphylococcal nuclease and bovine pancreatic ribonuclease, as well as several ribonuclease derivatives on columns of specific nucleotide inhitors bound to Sepharose 4B has permitted determination of nucleotide binding constants. Elutions are performed with varying concentrations of active site ligands and MLAB computer data fits are made to derived equations describing the competing equilibria between matrix bound nucleotide and free ligand for enzyme binding. In each case, the soluble ligand has been shown to effectively compete with matrix bound ligand. This affinity disruption by soluble ligand was shown to result in enzyme elution at a position inversely dependent on ligand concentration and directly so on its binding constant with the competing ligand. The determined elution volume, when used in derived equations, permits calculation of binding constants for both soluble and matrix bound ligands to the protein. Further, given a single affinity matrix, the binding constants of several ribonuclease derivatives could be determined. (Taylor, Chaiken)

II. Studies on proteins and nucleic acids employing interaction with antibodies

A. Immunochemical studies of tRNA

Studies have been carried out on tRNA bindingimmunoglobulins in the sera of NZB/NZW mice and in patients with systemic lupus erythematosus (SLE) as part of a project to obtain antibodies with conformational specificity for nucleic acids. The mice develop immunoglobulins which bind ^{3}H-tRNA, when they develop a disease process with similarities to SLE and we have been able to isolate these antibodies by affinity chromatography. Also studied were ^{3}H-tRNA binding immunoglobulins by these methods in the sera of several patients with SLE. This binding is better inhibited by single stranded viral RNA than by the tRNA itself. We examined changes which occur within a group of isoacceptor E. coli tRNAs before and after bacteriophage MS2 infection. Reversed phase column chromatography is different from that of virus infected E. coli leucyl-tRNA isoacceptors. The RPC-5 pattern of the latter tRNA shows several new peaks of leucyl-tRNA. Aminoacylation and codon recognition studies suggest that these tRNAs are some modified forms of normal leucine tRNA isoacceptors. This modification may be involved in the mechanism of inhibition of host protein synthesis by the virus.(Eilat, DiNatale, Schechter) (Cooperating Units: NIAMDD:A&R; NCI:CH; NIAID:LMI)

B. Interaction of antibodies with multideterminant antigens in radioimmunoassay: antibodies to sickle hemoglobin

Analyses of radioimmunoassay curves generally depend on the mass action law equations for simple equilibria between a single antigen and antibody. However, most natural protein antigens have multiple different antigenic determinants, and the antisera made to them are multispecific. The behavior of these systems is too complex to solve in a general analytic formulation, so that only individual cases can be treated numerically by computer. We have now developed a general theory for radioimmunoassay binding curves of multideterminant antigens, using probability theory. The only assumptions are that the determinants be unique and independent in their binding to antibodies. The predictive ability of the theory has been demonstrated for antibodies to subregions of the N-terminal third of the β chain of sickle hemoglobin, studied using antisera fractionated on affinity chromatographic columns of synthetic peptides. One implication is that to obtain quantitative binding parameters, such as affinity constants for multideterminant antigens, one should fractionate the sera to obtain monospecific antibodies. (Berzofsky, Curd, Schechter)

C. Monospecific antibodies as probes of hemoglobin structure and function

Using non-precipitating monospecific antibodies as probes of conformation, we are studying structural differences of hemoglobin in solution. Hemoglobin A_o and A_2 have been isolated, purified and carbamoylated with $KC^{14}NO$. Two specific subpopulations of anti-hemoglobin antibodies have been isolated by affinity chromatography using synthetic peptides corresponding to $\alpha(129\text{-}141)$ and $\delta(1\text{-}13)$ attached to a Sepharose support. The first subpopulation will be used to study conformational changes at the carboxyterminus of the α chain during oxygenation based on the difference in the binding affinity between C^{14} oxy and C^{14} deoxy hemoglobin. Differences will be used to examine mutant and truncated hemoglobins

so as to better understand the structural changes occurring during the dynamic transition from the T (deoxy) state to the R (oxy) state. The second subpopulation will be used to measure hemoglobin A_2 levels in serum and to differentiate A_2 from other normal and mutant hemoglobins. (Dean, Eastlake, Schechter)

D. Radioimmunoassay of creatine kinase: a model for radioimmunoassay of human serum enzymes

Human serum enzyme assays for clinical diagnosis usually make use of measurements of catalytic activity. In many situations, this may not be ideal; e.g., isoenzymes reflecting different damaged tissues may be indistinguishable or inactive enzymes may be released. Radioimmunassay offers a very sensitive and specific way in which to measure enzyme concentration and to distinguish isoenzymes. Creatine kinase was chosen as a model for this approach because of its clinical importance and its simple isoenzyme distribution. The enzymes from human skeletal muscle and brain have been purified, used as immunogens to raise antibodies, and labeled with ^{125}I. Radioimmunoassays for each of these enzymes have been developed and are now being applied in clinical diagnostic studies. (Schechter) Cooperating Unit: CC:CP

E. Functional properties of immune response gene regulation of the antibody response to nuclease

The ability to raise an immune response to specific antigens has recently been shown to be under genetic control, linked to the major histocompatibility complex (MHC) in several species studied. In the mouse, a number of synthetic antigens have been found subject to this H-2 linked control, but only a few natural globular proteins have been studied as antigens, among these, staphylococcal nuclease. We have now tried to delineate the individual antigenic determinants on this protein which are responsible for this genetic control of the immune response in inbred strains of mice, in order to better understand the mechanisms involved. We have found that a single peptide fragment of the protein appears to be under the same control as the whole molecule Furthermore, when antibodies made to the whole nuclease molecule are studied for their specificity, we have found that the H-2 linked immune response genes can control the response to individual determinants on the same molecule independently of one another, and therefore may be involved in the selection of specific B lymphocytes rather than only T cells. (Berzofsky and Schechter) Cooperating Unit: NCI:I

F. Immunological and structural studies of human fetal hemoglobin

Human fetal hemoglobin (HbF) has a weaker affinity for 2,3 diphosphoglyceric acid than HbA and other characteristics, such as non-participation in gel formation with HbS, that distinguish it from HbA. The molecular basis of these differences is not clear. HbF contains two α chains and two γ chains instead of two α chains and two β chains of HbA. As a result there are 39 amino acid differences between HbF and HbA, several of which are in the 2,3 diphosphoglyceric acid binding site. A number of others are clustered in one exposed helical region of the γ chain. We are using solid phase synthetic methods to reproduce this helical region. Immunoabsorption of an antiserum to HbF will then be used in an attempt to obtain non-precipitating antibodies specific for this region which do not crossreact with HbA. A radioimmunoassay

using [^{14}C]-carbamoylated HbF will be used to assess the specificity of binding. We have also obtained crystals of HbF of suitable size for X-ray diffraction studies. The specific antibodies and X-ray studies will give information on the conformational and surface structural characteristics of HbF. In addition the antibodies may be useful in quantitating small amounts of HbF in standard laboratory procedures and in prenatal diagnosis of hemoglobinopathies. (Eastlake, Thomas, Schechter)

G. Purification and characterization of rabbit antibodies against bovine neurophysin and their use for conformational and biosynthetic studies

Purified bovine neurophysins I and II were coupled specifically to succinylated polyalanyl-polylysine via protein amino groups. The resulting conjugates displayed essentially the same binding characteristics for the vasopresin-related ligand methionyl-tyrosyl-phenylalanine-amide (MTP) as the unmodified neurophysins. The conjugates elicited neurophysin-specific antibodies in rabbits. These antibodies are being isolated by affinity chromatography on neurophysin-Sepharose. They will be characterized and used first to study their interaction with the complex neurophysin-MTP and second to make immunoadsorption columns to isolate the postulated neurophysin precursor from hypothalmic tissue. Carbamoylated neurophysin I was found to be identical in its ligand binding properties to unmodified neurophysin. ^{14}C labelled neurophysin I (obtained by carbamoylation of NP with ^{14}C-cyanate) was used to develop a radioimmunoassay for the neurophysin antibodies using gel filtration on G100 for the separation of bound from free antigen. (Fischer and Chaiken)

III. Studies on protein folding and conformation involving complementing fragments or chains

A large number of proteins exist as polymers of a single subunit or, in some cases, of two or more different subunits. There are also a number of instances in which the fragments produced by cleaving the backbone chain of a globular protein will complement one another through noncovalent interactions to yield active complexes.

A. Studies of the kinetics of gelation of sickle cell hemoglobin: peptides as possible inhibitors

The gelation kinetics and solubility assay for deoxygenated hemoglobin S developed by Hofrichter, Ross and Eaton is being used to study the effect of amino acids, dipeptides and oligopeptides on gelation. The delay time of gelation is determined by monitoring turbidity changes at 800 nm. The solubility is determined from the supernatant infra-red spectra of the sample after the gel is sedimented at high speed in the ultracentrifuge. The anti-sickling effect of amino acids, dipeptides and oligopeptides can be determined by the amount of retardation of gelation and the increase of solubility of deoxygenated hemoglobin S. The ultimate goal of this project is to study the surface interactions of deoxygenated hemoglobin S and to find a therapeutic anti-sickling agent. Phenylalanine was the only amino acid of many tested that significantly affected gelation and solubility. The interpretation of these data will depend on the results of further studies investigating other amino acids. Current studies in progress also include investigating the dependence

of gelation and solubility of hemoglobin S on the concentration of amino acids (phenylalanine and tyrosine) and on the length of a homopolymer. When these basic data on the effects of amino acid and peptide additives are complete, we will begin a systematic study of the peptides from the contact regions which have been synthesized as possible stereospecific inhibitors of gelation. If indicated, studies of erythrocyte permeability and physiology (oxygen affinity, pH, etc.) will be done with selected peptides. (Noguchi, Eastlake, Schechter)

B. Protein unfolding by fragment exchange with a system derived from cytochrome c

The complex formed from the ferric fragment (1-65)H of cytochrome c and apocytochrome c was treated by limited digestion with trypsin in order to remove the redundant residues. The components of the derived complex were separated by ion exchange chromatography in 8 M urea. The three major non-heme and two major heme fragments were tentatively identified as (40-104), (54-104), (39-104), 1-53)H and (1-55)H. Measurements of the fluorescence quenching of tryptophan 59 of the non-heme peptides by the ferric peptides indicated that a 1:1 complex is formed in all 6 cases with a dissociation constant of less than 3×10^{-7} M. The complex (40-104):(1-53)H, which is obtained in the largest amount, has a K_{diss} $<3 \times 10^{-8}$ M and approximately 30% of the activity of native cytochrome c when reduced by lactate dehydrogenase from Baker's yeast. Thus cytochrome c can be cleaved at specific sites between residues 39 and 55 and reconstituted as a non-covalent complement with physical and biological properties similar to the parent molecule. This region containing the major sites of permissible trypsin cleavage is the same as that found by R.E. Dickerson et al. to have been excised in Pseudomonas cytochrome c_{551} during the course of evolution. (Hantgan and Taniuchi)

C. Studies on the relationship between the amino acid sequence and the three-dimensional structures of proteins

The equilibration of the system involving two alternative, enzymically active complementing structures, type I and II simultaneously formed from two overlapping fragments, Nuclease-(1-126) and Nuclease-T-(6-49) of Staphylococcal nuclease has been studied by determining the ratio of type I to type II complex as a function of incubation time, temperature and the presence or absence of ligands. The ratio of type 1 to type II complex initially formed was approximately 0.3 and independent of temperature and the presence or absence of ligands. The ratio of type I to type II complex after the two complexes have reached the equilibrium state through unfolding and folding is 1.1 and 2.4 at 6 and 23°, respectively. It is concluded that the rate of folding is not related to the decrease in energy from the unfolded to the folded state. A precursor of nuclease Foggi is found to contain the extra amino acid sequence of Ser-Glx-Thr-Asp-Asx-Gly-Val-Asx-Arg-Ser-Gly-Ser-Glu-Asp-Pro-Thr-Val-Tyr at the NH_2 terminus of nuclease. Nuclease-(1-126) saturated with substrates is shown to have approximately one-thousandth the enzymic activity of nuclease. Synthesis of Nuclease-T-(6-48) is being carried out by Dr. Carlo DiBello, University of Padova, Italy, in collaboration for the project of crystallization of semisynthetic Nuclease-T. The objective of this particular approach to the study of interatomic interactions is that measurements of the interaction energy maintaining the three-dimensional structure by the

method developed in our laboratory be combined with the X-ray crystallographic studies of semisynthetic Nuclease-T with substituted residues in order to understand quantitatively the contribution of various residues. (Taniuchi, Parker, Davis) Cooperating Units: NIAMDD:LMB; Institute of General Chemistry, University of Padova.

D. Functional and physicochemical characterization of bovine neurophysin-neurohypophyseal hormone complexes

Equilibrium dialysis experiments have shown that the vasopressin analogue, methionyl-tyrosyl-[^{14}C]phenylalaninamide, interacts with each of bovine neurophysins I and II specifically, having characteristics of binding similar to, but distinguished in important ways from, those of the intact peptide hormones. The analogue peptide serves as a valuable model and has allowed the preparation of an affinity matrix, to be used in future studies of neurophysin-hormone interacting complexes. Neurophysins were found to be highly susceptible to inactivation by disulfide interchange. Combined with the observation of reduction of this susceptibility by the ligand Met-Tyr-Phe amide, the results are consistent, among other possibilities, with the hypothesis that the neurophysins are biosynthesized as part of a larger precursor protein and that part of the additional polypeptide of this precursor may correspond to sequences analogous to those of the neurohypophyseal peptide hormones. (Chaiken and Taylor)

E. Study of protein conformation and biological function with semisynthetic noncovalent peptide-protein complexes

Analogues of semisynthetic ribonuclease-S' have been made for active site and conformationally important residues in the (1-20) fragment by solid phase peptide synthesis. Analogues for the α-helical position 9 have revealed critical correlations between the experimentally observed propensity for α-helix formation and empirical propensity parameters (generated by several groups by examination of proteins of known sequence and conformation). Additionally, analogues at the active site His 12 position have been made containing carbon 13 and fluorine 19 atoms. Such analogues, studied by proton and carbon 13 NMR, have helped refine the view of the participation of His 12 in ribonuclease catalysis and, in the case of the enzymically inactive 4-F-His 12 derivative, have provided a vehicle for future studies of the binding of substrates at the active site. Crystalline (normal sequence) semisynthetic ribonuclease-S' has been prepared and X-ray diffraction analyses indicate the structural identity to native ribonuclease-S'. Study of the interaction of bovine neurophysin II with ^{13}C-enriched synthetic oxytocin allowed inferences to be drawn concerning the mode of interaction of the neurohypophyseal hormone with its carrier protein. (Chaiken, Pandin, Taylor) (Cooperating units: NIAMDD: LMB, LCB, LC)

F. Studies of the mechanism of native pairing of half-cystine residues in proteins containing disulfide bonds

The material obtained from reduced hen egg white lysozyme after complete air oxidation was fractionated by gel filtration to yield the enzymically active, native species and the enzymically inactive form which eluted at an elution volume smaller than that of the native species but greater than that expected

for a dimeric form of lysozyme. The yield of the inactive form increased up to 100% when the oxidation of reduced lysozyme was accelerated using cupric ion. This inactive form, shown to contain incorrect disulfide bonds, can be quantitatively renatured by sulfhydryl-disulfide interchange. In the early phase of oxidation of reduced lysozyme three partially oxidized, active species were trapped by alkylation with ^{14}C-iodoacetate. Isolation and characterization of the radioactive tryptic peptides from each of the 3 active forms, permitted the identification of Cys-6 and Cys-127, Cys-76 and Cys-94, and Cys-80, respectively, as the free sulfhydryl groups in the three incompletely oxidized species. Thus, these active species appear to contain three native disulfide bonds and one open disulfide bond. Two of these active species containing alkylated sulfhydryl groups at residues 6 and 127 and at residues 76 and 94, respectively, appear to be capable of renaturing after reduction and reoxidation. Trapping, by alkylation, the active species containing sulfhydryl groups formed during sulfhydryl-disulfide interchange of the inactive species and examination of the radioactive tryptic peptides have suggested that the active species containing three native disulfide bonds and one open disulfide bond can be formed in all possible cases. (Acharya and Taniuchi)

IV. Isolation and characterization of proteins

A. Isolation, purification and chemical characterization of the human neurophysins

Acetone powder of human posterior pituitary lobes was acid extracted and the neurophysins isolated. An affinity chromatography system was developed in order to simplify the isolation and to allow quantitative binding studies. This system consists of a vasopressin analogue, L-methionyl-L-tyrosyl-L-phenylalanine, attached through an 8 A side arm to Sepharose 4B. Neurophysins are retained on this column through the pH range 4.5 to 6.9. One component of the neurophysin fraction, not removed by any other step of the isolation procedure, is separated through use of this affinity system. The binding properties of the neurophysins will be investigated with equilibrium dialysis and quantitative affinity chromatography. Immunologic similarities between human and bovine neurophysins will be studied with antibodies raised against bovine neurophysins. (McCormick and Chaiken) (Cooperating unit: National Pituitary Agency)

B. Isolation and characterization of immobilization antigen from *P. aurelia*

The immobilization antigen (i-antigen) is a surface protein of the protozoan P. aurelia. In the presence of homologous antisera made against the i-antigen, the animals become immobilized and eventually die. Although the exact function of this protein is unknown, the i-antigen is of interest due to the unusual mechanism controlling its expression. Only one of as many as 12 i-antigens is believed to be produced by any one animal at any given time and this synthesis is affected by the state of the cytoplasm and the environment of the animal. Some knowledge of the i-antigen itself and a comparative analysis of the various i-antigens, will greatly aid in the understanding of the mechanisms involved in inter- and intra-cellular differentiation. A controversy as to the exact quaternary structure and make-up of the i-antigen

has arisen because the methods previously used to purify the i-antigen have been shown to yield contaminating proteins, some of which contain degradative activity. We have used ion exchange chromatography on DEAE-Sephadex and SE-Sephadex and affinity chromatography (with specific antibodies attached to Sepharose) to purify the i-antigen. This purified preparation will be used to physically and chemically characterize the i-antigen. (Steers and Davis)

C. Isolation, structural determination, and synthesis of human interferon

Interferon is a cellular protein capable of suppressing the replication of an infecting virus within the invaded animal cell. It generally exhibits antiviral activity in cells of the same or closely related species. The main objective of this project is the isolation, structural determination and synthesis of human interferon. The synthesis of interferon appears to be the only practical approach to a thorough clinical trial of this antiviral substance since it is present in minute amounts in biological sources. The specific activity of interferon in biological fluid is very low. Therefore, an attempt was made to prepare radioactive human fibroblast, leukocyte and lymphoblastoid interferons. Human fibroblast, leukocyte and lymphoblastoid cell cultures, induced with poly I-poly C or Sendai virus, were incubated in the presence of radioactive amino acids. Any given incubation involved only two or three tritium labeled radioactive amino acids. Following purification by affinity chromatography, gel filtration, ion exchange chromatography, isoelectric focusing and slab gel electrophoresis, the isolated minute amounts of radioactive interferon were subjected to sequence analysis together with apomyoglobin as a carrier protein using a highly sensitive microsequencing technique developed in connection with this research project. The phenylthiohydantoin was identified, either by high pressure liquid chromatography, or by thin layer chromatography on polyamide plates and radioautography. The results obtained with tritiated interferon were inconclusive because of insufficient radioactivity. We are currently engated in the large scale production of lymphoblastoid interferon in conjunction with the Frederick Cancer Research Center, Frederick, Maryland. Interferon has been successfully induced in 5-7 liter cell cultures using Sendai or NDV-B1 virus. Operations are in progress to scale up the production of interferon stepwise through 7, 30, 300, 2200 gallon volumes.
(Anfinsen, Bose, Corley, Gurari-Rotman, Ruegg, Zoon)
(Cooperating units: Cancer Research Center, Frederick, Md.; NIAID:LVD)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,000-03 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Isolation, Structural Determination, and Synthesis of Human Interferon

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

PI:	C.B. Anfinsen	Chief, Laboratory of Chemical Biology	LCB NIAMDD
	S. Bose	Special Fellow	LCB NIAMDD
	L. Corley	Chemist	LCB NIAMDD
	D. Gurari-Rotman	Visiting Scientist	LCB NIAMDD
	U. Th. Ruegg	Visiting Fellow	LCB NIAMDD
	K. Zoon	NIAID Fellow	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	4.5	1.0

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

Leukocyte, fibroblast and lymphoblastoid interferons were removed from crude tissue culture fluids by columns of anti-leukocyte interferon attached to Sepharose-4B.

As specific activity of interferon in biological fluid is very low, attempts were also made to prepared radioactive interferons in tissue culture medium. Following purification by affinity chromatography, gel filtration, ion exchange chromatography, isoelectric focusing and slab gel electrophoresis, the isolated minute amounts of radioactive interferon were subjected to sequence analysis. The results obtained with tritiated interferon were inconclusive because of insufficient radioactivity.

Currently, we are engaged in the large scale production of lymphoblastoid interferon in conjunction with the Frederick Cancer Research Center, Frederick, Md. Following the induction of interferon with NDV-B1 virus lymphastoid cells are removed from the medium by filtration using an array of filters and Hyflo-Super-Cel. The virus present in the filtrate is killed by boiling for 10 minutes at pH 2. The conditions for the concentration of interferon by SDS_{ppt} are in progress in this laboratory.

PHS-6040
(12-75)

Project Description:

Objectives: Interferon is a cellular protein capable of suppressing the replication of an infecting virus within the invaded animal cell. It generally exhibits antiviral activity in cells of the same or closely related species.

The main objective of this project is the isolation, structural determination and synthesis of human interferon. The synthesis of interferon appears to be the only practical approach to a thorough clinical trial of this antiviral substance since it is present in minute amounts in biological sources.

Methods Employed and Major Findings: Leukocyte, fibroblast and lymphoblastoid interferons were removed from crude tissue culture fluids by columns of anti-leukocyte interferon attached to Sepharose-4B. The antibody was prepared in sheep using, as antigen, material that had been partially purified by gel filtration through Sephadex-G100 column. Anti-interferon in the γ-globulin fraction was purified before attachment to Sepharose by passage through an affinity column bearing the protein impurities that might be present in the crude interferon-containing culture fluid (serum proteins and proteins of the buffy coat cells, allantoic fluid, and Sendai virus). The results of the early part of our work on the purification of interferon have been summarized in Proc. Nat. Acad. Sci. 71, 3139, 1974.

The specific activity of interferon in biological fluid is very low. Therefore an attempt was made to prepare radioactive human fibroblast, leukocyte and lymphoblastoid interferons. Human fibroblast, leukocyte and lymphoblastoid cell cultures, induced with poly I-poly C or Sendai virus, were incubated in the presence of radioactive amino acids. Any given incubation involved only two or three tritium labelled radioactive amino acids.

Following purification by affinity chromatography, gel filtration, ion exchange chromatography, isoelectric focusing and slab gel electrophoresis, the isolated minute amounts of radioactive interferon were subjected to sequence analysis together with apomyoglobin as a carrier protein using a highly sensitive microsequencing technique developed in connection with this research project. The phenylthiohydantoin was identified either by high pressure liquid chromatography, or by thin layer chromatography on polyamide plates and radioautography.

The results obtained with tritiated interferon were inconclusive because of insufficient radioactivity.

We are currently engaged in the large scale production of lymphoblastoid interferon in conjunction with the Frederick Cancer Research Center, Frederick, Md. Interferon has been successfully induced in 5-7 liter cell cultures using Sendai or NDV-B1 virus. Operations are in progress to scale up the production of inerferon stepwise through 7, 30, 300 and 2200 gallon volumes. Following the induction of interferon, the lymphoblastoid cells are removed from the medium by filtration using an array of filters and Hyflo-Super-Cel. The filtrate is adjusted to pH 2 and boiled (100°C/10 min). The first step in the purification is the concentration of the interferon. This is important particularly at the larger scales for it will be necessary to reduce the volume of the interferon containing supernatant to a quantity which can be processed in the laboratory.

We are examining several precipitation techniques including sodium dodecyl sulfate at acid pH, picric acid, and zinc acetate. The concentrated interferon will then be further purified using gel filtration, affinity and ion exchange chromatography, isoelectric focusing and SDS polyacrylamide gel electrophoresis. To determine the complete sequence of the interferon molecule, the purified labeled interferon will be fragmented two different ways to produce overlapping peptides using: 1) trypsinization of succinylated interferon which produces COOH - terminal arginine peptides; and 2) cyanogen bromide treatment of the purified interferon, which results in the formation of COOH - terminal homoserine lactone peptides. These peptides will then be separated by chromatography and sequenced on an automated Edman sequencer. By comparing the amino acid composition of the two sets of overlapping peptide fragments, the correct sequence can be obtained.

Once the sequence has been determined, the chemical synthesis of the polypeptide portion of the interferon molecule will be attempted using a combination of the Merrifield solid-phase technique and classical fragment synthesis methods.

The carbohydrate components of fibroblast interferon have been studied using radioactive glucosamine incorporation to label the sugar moieties. Digestion of such radioactive interferon with a broad spectrum of glycosidases 9an enzyme-mixture consisting of neuraminidase, β-galactosidase, α-mannosidase, N-acetyl glucosaminidase, etc.) removes 85-90% of the labelled sugar without loss of antiviral activity. At the same time the interferon loses much of its heterogeneity, presumably due to variable contents of sialic acid. Dorner et al. have shown that the terminal sequence of the carbohydrate moiety attached to the polypeptide chain of interferon is sialic acid-galactose and yields one fairly sharp zone upon electrofocusing. On treatment with glycosidases, the molecular weight of leukocyte interferon was reduced by about 4×10^3. (This part of our work on interferon has been summarized in J. Biol. Chem. 251, 1659, 1976.)

Since our recent work on interferon shows that the antiviral activity of interferon does not depend on the carbohydrate moiety attached to its polypeptide chain, there is a great possibility that the chemically synthesized interferon will exhibit antiviral activity even without having its carbohydrate moiety.

The purification of interferon and its sequencing will be continued using a large scale production method and if that is successful, studies will be initiated to synthesize interferon and determine the antiviral acitvity and the rate of catabolism of this molecule compared to the control purified interferon.

Publication:

Bose, S., Gurari-Rotman, D., Ruegg, U.Th., Corley, L. and Anfinsen, C.B.: Apparent dispensability of the carbohydrate moiety of human interferon for antiviral activity. J. Biol. Chem. 251, 1659-1662, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,001-02 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of Immobilization Antigen from P aurelia

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

PI:	E. Steers Jr.	Research Biologist	LCB NIAMDD
	R.H. Davis Jr.	Guest Worker	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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

The immobilization antigen (i-antigen) is a surface protein of the protozoan P. aurelia. In the presence of homologous antisera made against the i-antigen, the animals become immobilized and eventually die. Although the exact function of this protein is unknown, the i-antigen is of interest due to the unusual mechanism controlling its expression. Only one of as many as 12 i-antigens is believed to be produced by any one animal at any given time and this synthesis is affected by the state of the cytoplasm and the environment of the animal. Some knowledge of the i-antigen itself and a comparative analysis of the various i-antigens, will greatly aid in the understanding of the mechanisms involved in inter- and intra-cellular differentiation. A controversy as to the exact quaternary structure and make-up of the i-antigen has arisen because the methods previously used to purify the i-antigen have been shown to yield contaminating proteins, some of which contain degradative activity. We have used ion exchange chromatography on DEAE-Sephadex and SE-Sephadex and affinity chromatography (with specific antibodies attached to Sepharose) to purify the i-antigen. This purified preparation will be used to physically and chemically characterize the i-antigen.

PHS-6040

Project Description:

Objective: To purify and characterize the immobilization antigen from P. aurelia.

Methods Employed:
1) Purification-chromatography on DEAE and SE-Sephadex. Chromatography on anti-i-antigen-Sepharose (affinity chromatography). The purification procedure was followed by standard 7 1/2%; SDS; 8 M urea acrylamide disc gel electrophoresis and by immunodiffusion in agar gels.
2) Characterization-disc gel electrophoresis, ultracentrifugation, fingerprinting and amino acid analysis.

Major Findings: The i-antigen purified by previously published methods is not pure. Electrophoresis of these preparations on 7 1/2% standard, SDS or 8 M urea gel electrophoresis shows multiple components. In the presence of reducing agents such as dithiothreitol, degradative activity can be demonstrated on SDS and 8 M urea acrylamide gel electrophoresis. On DEAE-Sephadex, at pH 7.0, the degradative activity was separated from the immunological activity in these preparations. The void volume fraction (D-I), which contained the immunological activity, was also found to contain several components on disc gel electrophoresis. D-I was applied to SE-Sephadex in 0.05 M acetate buffer at pH 4.2. The stepwise elution with acetate buffer at pH 4.6 and 5.2 allowed the separation of 4 fractions from this column (S-I, S-II, S-III and S-IV). The separated fractions were used to immunize rabbits; the major fraction S-II or the anti-S-II serum was found to be the only serum to immobilize the standard type 51A paramecium.

Ion exchange chromatography proved to be impractical as a preparative technique. Less than 50% of the applied protein could be eluted from the gel and a major portion of the elution peak of S-II was contaminated by S-III. Therefore affinity chromatography utilizing anti S-II antibodies attached to CNBr activated Sepharose was utilized. This affinity column was specific for S-II and did not bind BSA, S-III or S-IV. The anti-S-II-Sepharose column binds S-II in 0.05 M PO_4 pH 7.3 and elutes the bound material in 0.05 M glycine pH 10.4 buffer or 6 M urea in 0.05 M phosphate buffer. The protein applied eluted quantitatively.

The i-antigen purified in this manner gave a single component on standard 7 1/2%; SDS and 8 M urea acrylamide gel electrophoresis. This purified preparation will be used to further physically and chemically characterize the immobilization antigen.

Significance to Biomedical Research: The immunologic specificity of the protozoan Paramecium has been shown to be the result of a surface associated protein (immobilization antigen) whose antigenic specificity is under the control of cytoplasmic and nuclear determinants.

Although researchers agree that the protein responsible for the immobilizing reaction in paramecium is a large, fiberous protein of approximately 300,000 daltons, there is conflicting evidence concerning the existence and number of subunits composing the protein. The existence or lack of subunit

structure will give insight on the gene(s) responsible for their production and aid in understanding the cross-reactions previously observed in gel diffusion test and the method(s) by which this protein is regulated. The investigation of intracellular differentiation should give insights and contribute to the understanding of intercellular differentiation.

Proposed Course: The physical and chemical characterization of the i-antigen will involve 1) ultracentrifugation in phosphate buffer, 8 M urea and 8 M urea + dithiothreitol of natural and reduced carboxymethylated i-antigen; 2) fingerprinting of CNBr and enzymatically cleaved i-antigen; 3) amino acid analysis. These findings will be compared to previously reported data on impure i-antigen. These manipulations and investigations should indicate the exact nature of the i-antigen.

Publication:

Steers, E. and Davis Jr., R.H.: A Reexamination of the Immobilization Antigen I. Comparative Biochem. & Physiol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 25,002-03 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Isolation and Characterization of Myoglobin From Paramecium

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

Principal Investigators:

Edward Steers, Jr.	Research Biologist	LCB NIAMDD
Richard H. Davis, Jr.	Guest Worker	LCB NIAMDD

COOPERATING UNITS (If any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIAMDD:NIH; Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0		

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

The protozoan Paramecium aurelia has been shown to contain a respiratory pigment which appears homologous to mammalian myoglobin. This pigment is present in five electrophoretically different forms with isoelectric points of 5.54, 5.18, 4.98, 4.51 and 4.16 respectively. Purification of these pigments involves ammonium sulfate fractionation, gel filtration through Sephadex G-75 and G-50 and electrophoresis employing either polyacrylamide gels or isoelectric focusing. The molecular weights of the five pigments is approximately 16,000. The physiological role of these pigments remains obscure at this time. Whether the electrophoretic forms show functional heterogeneity or are functionally homogeneous is also not known at this time.

Work on this project is in abeyance at the present time. It will be resumed in the near future.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,003-06 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Study of Protein Conformation and Biological Function with Semisynthetic Noncovalent Peptide-protein Complexes

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

PI:	I.M. Chaiken	Research Chemist	LCB NIAMDD
	M. Pandin	Visiting Fellow	LCB NIAMDD
	H.C. Taylor	Chemist	LCB NIAMDD

COOPERATING UNITS (if any)
1. Laboratory of Molecular Biology, NIAMDD (Eduardo A. Padlan)
2. Physical Sciences Laboratory, NIAMDD (Jack S. Cohen)
3. Laboratory of Chemistry, NIAMDD (Louis A. Cohen and Kenneth L. Kirk)
4. Laboratory of Chemistry (Edward A. Sokoloski)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	1.5	.4

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

Analogues of semisynthetic ribonuclease-S' have been made for active site and conformationally important residues in the (1-20) fragment by solid phase peptide synthesis. Analogues for the α-helical position 9 have revealed critical correlations between the experimentally observed propensity for α-helix formation and empirical propensity parameters (generated by several groups by examination of proteins of known sequence and conformation). Additionally, analogues at the active site His 12 position have been made containing carbon 13 and fluorine 19 atoms. Such analogues, studied by proton and carbon 13 NMR, have helped refine the view of the participation of His 12 in ribonuclease catalysis and, in the case of the enzymically inactive 4-F-His 12 derivative, have provided a vehicle for future studies of the binding of substrates at the active site. Crystalline (normal sequence) semisynthetic ribonuclease-S' has been prepared and X-ray diffraction analyses indicate the structural identity to native ribonuclease-S'. Study of the interaction of bovine neurophysin II with ^{13}C-enriched synthetic oxytocin allowed inferences to be drawn concerning the mode of interaction of the neurohypophyseal hormone with its carrier protein.

PHS-6040
(12-75)

Project Description:

Objectives:

1) To define general approaches for the use of semisynthesis in the study of interacting peptide-protein systems.

2) To prepare, by chemical synthesis, polypeptide and protein fragments which interact with complementary native proteins or protein fragments to form biologically active complexes.

3) To purify semisynthetic derivatives in a state suitable for detailed conformational and functional characterization.

4) To examine the influence of side-chain characters on peptide-protein interaction, conformational stability, and function.

Methods Employed: A specific protein fragment, ribonuclease-S-(1-20) has been studied with respect to the noncovalent, enzymically active ribonuclease-S complex formed upon addition to the complementary fragment ribonuclease-S-(21-124). Semisynthetic analogues of the native complex have been obtained by chemical synthesis (solid-phase procedure) of ribonuclease-S-(1-20) and -(1-15) variants. With the latter, the microenvironments of specifically chosen loci have been studied, using spectroscopic (carbon 13 and proton NMR, absorption spectroscopy) and enzymic methods. X-ray diffraction analyses were performed on crystalline normal sequence [1-20 peptide]SRNase S' prepared at pH 5.3. Solid phase peptide synthetic oxytocin derivatives have been prepared and their binding to Neurophysin II studied by competition assays and carbon 13 NMR.

Major Findings: Crystals of solid phase derived semisynthetic ribonuclease S' were prepared and compared structurally with crystals of ribonuclease-S' and -S. This semisynthetic complex was completely active enzymatically and was homogeneous as judged by polyacrylamide gel electrophoresis. Crystallization of both semisynthetic and native ribonuclease-S' resulted in well-formed crystals with symmetry of space group $P3_121$ and unit cell dimensions a-b-44.82, c-97.3 Å. This crystal form corresponds to the Y form of native ribonuclease-S previously reported. X-ray diffraction patterns of the crystal types were indistinguishable, indicating the structural identity of semisynthetic and native ribonuclease-S' in crystal form.

Significance to Biomedical Research: The above studies provide insight into the potential use of semisynthesis in studying and designing peptide-protein systems of biomedical (including therapeutic) interest. The specific results of this work should allow a more complete understanding of the structural bases for conformation and biological function of protein-containing systems in general.

Proposed Course of Action:

1) Preparation, purification and crystallization of several semisynthetic ribonuclease-S' analogues.

2) Study of several of the ribonuclease-S analogues already prepared such as [4-F-His^{12}]Semisynthetic ribonuclease-S', both in the crystal form and in solution.

3) Design of further derivatives of semisynthetic noncovalent peptide-protein complexes for the study of other loci, in a program aimed at eventually defining rules of amino acid function.

Publications:

Chaiken, I.M. and Dunn, B.M.: Semisynthetic ^{13}C-Enriched and ^{19}F-Labelled Peptide-protein Noncovalent Complexes. Proceedings of the 13th European Peptide Symposium, Peptides 1974. pp. 299-309.

Dunn, B.M. and Chaiken, I.M.: Relationship Between α-helical Propensity and Formation of the Ribonuclease-S Complex. J. Mol. Biol. 95, 497-511, 1975.

Pandin, M., Padlan, E.A., DiBello, C. and Chaiken, I.M.: Crystalline Semisynthetic Ribonuclease-S. Proc. Nat. Acad. Sci., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,004-03 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Quantitative Affinity Chromatography

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

PI:	H.C. Taylor	Chemist	LCB NIAMDD
	I.M. Chaiken	Research Chemist	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.2	0.6

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

Affinity chromatography of staphylococcal nuclease and bovine pancreatic ribonuclease as well as several ribonuclease derivatives on columns of specific nucleotide inhibitors bound to Sepharose 4B has permitted determination of nucleotide binding constants. Elutions are performed with varying concentrations of active site ligands and MLAB computer data fits are made to derived equations describing the competing equilibria between matrix bound nucleotide and free ligand for enzyme binding.

PHS-6040
(12-75)

Project Description:

Objectives:

1) To continue development of methods using affinity chromatography to determine ligand binding constants.

2) To expand application of this method to study of the binding parameters of protein systems with more complex equilibria and/or altered biological activity.

3) To better understand general principles of affinity chromatography of macromolecules.

Methods Employed: Chromatography of highly purified staphylococcal nuclease was performed on columns of deoxythymidine-5'-phosphate-3-(p-aminophenyl) phosphate-Sepharose, in the presence of various concentrations of competing active site ligand, deoxythymidine-3',5'-diphosphate. Elution behavior on the above affinity matrix, and others prepared by dilution with unsubstituted Sepharose 4B, has been compared to behavior predicted by equations derived from first principles.

More recently, highly purified bovine pancreatic ribonuclease has been chromatographed on an affinity matrix of uridine-3'-phosphate-2'-(p-aminophenyl)phosphate-Sepharose in the presence of varying cytidine-2'-phosphate concentration, as well as varying protein concentration and ionic strength. Its elution behavior and that of ribonuclease derivatives, including Ribonuclease-S, [p-F-phe^{8} 1-15 peptide] SRNase-S' and [4-F-His^{12} 1-15 peptide] SRNase-S' and [normal 1-15 peptide]SRNase S, have been compared to the derived equations as well as our hypotheses about non-specific interactions and predictions of other researchers. Some semisynthetic ribonuclease complexes have been prepared which are tritium-labelled, facilitating detection of small amounts of protein eluted in the presence of a variety of nucleotide ligands. Results are computer fitted to derived equations to yield binding parameters.

Major Findings: In each case, the soluble ligand has been shown to effectively compete with matrix bound ligand. This affinity disruption by soluble ligand was shown to result in enzyme elution at a position inversely dependent on ligand concentration and directly so on the binding constant (K_d, as determined by other methods) with the competing ligand. The determined elution volume, when used in derived equations, permits calculation of binding constants for both soluble and matrix bound ligand to the protein. Further, given a single affinity matrix, the binding constants of several ribonuclease derivatives could be determined. Elution volume of protein with a given concentration of nucleotide was found to depend on protein concentration in a manner consistent with theoretical predictions of other groups. Elution volume at a given nucleotide concentration also decreased with an increase of ionic strength to a point where related but non-binding proteins were no longer non-specifically retarded, then remained constant with further increases in ionic strength.

Significance to Biomedical Research: Application of affinity chromatographic methods will readily facilitate determination of a binding constant

for any ligand, or possibly significant ligand derivative, to any protein or derivatized protein. It would provide an alternative to often imprecise or impracticable bioassays for hormone (or other ligand) binding to protein. Studies of the interaction between peptide derivatives and complementary protein fragments could yield important information about the effect on structural integrity, hence, on biological activity, of biologically altered enzymes and binding proteins.

Proposed Course of Project:

1) Further binding constant determinations for different protein semi-synthetic derivatives and inhibitors in the bovine pancreatic ribonuclease system.

2) Extended application to other cases including important peptide-protein and protein-protein systems.

Publications:

Dunn, B.M. and Chaiken, I.M.: Evaluation of Quantitative Affinity Chromatography by Comparison with Kinetic and Equilibrium Dialysis Methods for the Analysis of Nucleotide Binding to Staphylococcal Nuclease. Biochemistry 14, 2343-2349, 1975.

Chaiken, I.M. and Taylor, H.C.: Quantitative Affinity Chromatography and its Application to Studies of Ligand Binding by Semi-synthetic Ribonuclease-S' Analogues. In Walter, R. and Meienhofer, J. (Eds.) Peptides: Chemistry, Structure and Biology. Proceedings of the Fourth American Peptide Symposium. Ann Arbor Science Pub., 1975, pp. 1013-1020.

Chaiken, I.M. and Taylor, H.C.: Analysis of Ribonuclease-nucleotide Interactions by Quantitative Affinity Chromatography. J. Biol. Chem. 251, 2044-2048, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,005-03 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Functional and Physicochemical Characterization of Bovine Neurophysin-neurohypophyseal Hormone Complexes

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

PI:	I.M. Chaiken	Research Chemist	LCB NIAMDD
	H.C. Taylor	Chemist	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	Project is temporarily in abeyance, but will continue in the near future.	

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

Equilibrium dialysis experiments have shown that the vasopressin analogue, methionyl-tyrosyl-[^{14}C]phenylalaninamide, interacts with each of bovine neurophysins I and II specifically, having characteristics of binding similar to, but distinguished in important ways from, those of the intact peptide hormones. The analogue peptide serves as a valuable model and has allowed the preparation of an affinity matrix, to be used in future studies of neurophysin-hormone interacting complexes.

Sedimentation-equilibrium studies have shown that ligands affect aggregation properties in such a way as to suggest a relationship between the state of aggregation of the neurophysins and the quantitative character of the interaction with hormones.

Neurophysins were found to be highly susceptible to invactivation by disulfide interchange. Combined with the observation of reduction of this susceptibility by the ligand Met-Tyr-Phe amide, the results are consistent, among other possibilities, with the hypothesis that the neurophysins are biosynthesized as a part of a larger precursor protein and that part of the additional polypeptide of this precursor may correspond to sequences analogous to those of the neurohypophyseal peptide hormones.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,006-03 LCB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Isolation, Purification and Chemical Characterization of the Human Neurophysins

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

PI:	W.M. McCormick	Chemist	LCB NIAMDD
OTHER:	I.M. Chaiken	Research Chemist	LCB NIAMDD

COOPERATING UNITS (if any)

National Pituitary Agency (associated with the University of Maryland School of Medicine and NIAMDD)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.2	0.8

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

Acetone powder of human posterior pituitary lobes was acid extracted and the neurophysins isolated. An affinity chromatography system was developed in order to simplify the isolation and to allow quantitative binding studies. This system consists of a vasopressin analogue, L-methionyl-L-tyrosyl-L-phenylalanine, attached through an 8 Å side arm to Sepharose 4B. Neurophysins are retained on this column through the pH range 4.5 to 6.9. One component of the neurophysin fraction, not removed by any other step of the isolation procedure, is separated through use of this affinity system.

The binding properties of the neurophysins will be investigated with equilibrium dialysis and quantitative affinity chromatography. Immunologic similarities between human and bovine neurophysins will be studied with antibodies raised against bovine neurophysins.

PHS-6040
(12-75)

Project Description:

The objective of this project is to isolate and purify the neurophysins from the posterior lobe of the pituitary so that the neurophysins can be studied structurally in order that we may better understand the binding and other functions of neurophysins in the body.

Methods: Isolation of neurophysins from human tissue obtained from the National Pituitary Agency has proceeded according to methods previously established for other mammalian species. Both crude and purified fractions were examined on an affinity chromatography system (L-methionyl-L-tyrosyl-L-phenylalanine attached with a peptide bond to diaminobutane Sepharose) which binds neurophysins analogously to the neurophysin-hormone interaction.

Continued investigation of the human neurophysins allows revision of earlier reported findings. Two major human neurophysins and three minor components have been eluted from the affinity system while a third major component which is co-isolated with the neurophysins through all other steps of the extraction and purification is separated from the neurophysins.

Significance to Biomedical Research: Study of the human neurophysins should be useful in determining their biological function. As more is learned of their peptide binding characteristics, a better understanding of protein-peptide interaction in general, should become apparent.

A better understanding of the mechanism of hypothalamic hormone synthesis, transport and controlled release may also be possible, all of which is important to understanding the functioning (on a molecular level) of the endocrine glands.

Proposed Course: The future course of this project includes further modification of the purification procedure including the use of affinity chromatography. Affinity chromatography will also be used as a quantitative tool for binding studies. Equilibrium dialysis results will be used to corroborate binding study results. Antibodies raised against bovine neurophysins will be used to compare antigenic properties of human and bovine neurophysins.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,007-03 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of Rabbit Antibodies Against Bovine Neurophysin and Their Use for Conformational and Biosynthetic Studies.

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

PI:	E.A. Fischer	Guest Worker	LCB NIAMDD
OTHER:	I.M. Chaiken	Research Chemist	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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

Purified bovine neurophysins I and II were coupled specifically to succinylated polyalanyl-polylysine via protein amino groups. The resulting conjugates displayed essentially the same binding characteristics for the vasopressin-related ligand methionyl-tyrosyl-phenylalanine-amide (MTP) as the unmodified neurophysins. The conjugates elicited neurophysin-specific antibodies in rabbits. These antibodies are being isolated by affinity chromatography on neurophysin-Sepharose. They will be characterized and used first to study their interaction with the complex neurophysin-MTP and second to make immunoadsorption columns to isolate the postulated neurophysin precursor from hypothalmic tissue. Carbamoylated neurophysin I was found to be identical in its ligand binding properties to unmodified neurophysin. ^{14}C labelled neurophysin I (obtained by carbamoylation of NP with ^{14}C-cyanate) was used to develop a radioimmunoassay for the neurophysin antibodies using gel filtration on G100 for the separation of bound from free antigen.

PHS-6040
(12-75)

Project Description:

In order to study the protein peptide interaction between neurophysin and the neurohypophyseal hormones antibodies against this protein could be useful for detecting eventual conformational changes. Additionally, they could be used to establish the residues involved in the actual binding event. Moreover, at least some of the antibodies should recognize the postulated neurophysin precursor and could therefore be used to isolate this species. As bovine neurophysins are weak immunogens in rabbits, we developed a method to couple them to polyalanyl-polylysine in order to increase their immunogenicity. The polymer carrier was succinylated; the resulting carboxyl groups converted into hydrazide and subsequently into azide groups and coupling was achieved by intermolecular reaction with protein amino groups. This procedure of chemically activating the synthetic polymer cannot introduce any unwanted modifications in the protein and yields biologically active conjugates. The neurophysin conjugates displayed essentially the same binding properties for the vasopressin related ligand methionyl-tyrosyl-phenylalanine amide (MTP) as the unmodified neurophysins (Binding studies by equilibrium dialysis). Bovine pancreatic ribonuclease, coupled to the polymer with the same method, yielded an enzymically fully active derivative.

The retention of biological activity of the proteins in the conjugates is very important in view of the planned use of the antibodies as conformational probes. It indicates that the proteins are very little disturbed conformationally in the conjugates and allows the conclusion that antibodies raised with the conjugates will recognize the unmodified proteins. This could be shown in competition experiments, where formation of precipitin lines with conjugate could be inhibited by the addition of the corresponding free neurophysin. The antisera were fractionated on Sepharose bound neurophysin from which a purified antibody fraction could be eluted with 6 M guanidine hydrochloride. The binding properties of the antibody fractions were monitored with a radioimmunoassay, using ^{14}C labelled neurophysin, which had been obtained through carbamoylation with ^{14}C-cyanate. (Extensive carbamoylation of neurophysin I does not affect its binding properties for MTP.) In this assay, bound and free neurophysin were separated by gel filtration on a G-100 column. Separation of bound from free neurophysin could also be achieved using a protein A carrying strain of staphylococcus aureus as the antibody adsorbent.

The methodology of specific protein-carrier immunogen preparation developed here may be useful in general for immunological studies of weak protein antigens. The studies undertaken should allow an increased understanding of the function of neurophysins as carrier proteins for neurohypophyseal hormones. They hopefully will cast light on the biosynthetic origin and fate of the neurohypophyseal hormones before entering the pituitary. The protein-hormone complex could also serve as a model for hormone-receptor interaction.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,008-13 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies on the Relationship Between the Amino Acid Sequence and the Three-dimensional Structures of Proteins: the Mechanism of Protein Chain Folding and Protein Functions

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

PI:	H. Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD
OTHER:	D.S. Parker	Chemist	LCB NIAMDD
	A. Davis	Chemist	LCB NIAMDD

COOPERATING UNITS (if any)
Section on Molecular Structure, Laboratory of Molecular Biology, NIAMDD
Institute of General Chemistry, University of Padova, Padova, Italy

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Conformation Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.4	1.0	2.4

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

1) The equilibration of the system involving two alternative, enzymically active complementing structures, type I and II simultaneously formed from two overlapping fragments, Nuclease-(1-126) and Nuclease-T-(50-149) of Staphylococcal nuclease has been studied by determining the ratio of type I to type II complex as a function of incubation time, temperature and the presence or absence of ligands. The ratio of type I to type II complex initially formed was approximately 0.3 and independent of temperature and the presence or absence of ligands. The ratio of type I to type II complex after the two complexes have reached the equilibrium state through unfolding and folding is 1.1 and 2.4 at 6 and 23°, respectively. It is concluded that the rate of folding is not related to the decrease in energy from the unfolded to the folded state.

2) A precursor of nuclease Foggi is found to contain the extra amino acid sequence of Ser-Glx-Thr-Asp-Asx-Gly-Val-Asx-Arg-Ser-Gly-Ser-Glu-Asp-Pro-Thr-Val-Tyr at the NH_2-terminus of nuclease.

3) Nuclease-(1-126) saturated with substrates is shown to have approximately one-thousandth the enzymic activity of nuclease.

Project Description:

Objectives: To understand interatomic interactions in the three-dimensional structures of proteins in relation to the structures and functions of proteins.

Working Hypothesis: A working hypothesis has been developed on the basis of the studies of the complementing fragments of staphylococcal nuclease and des-(121-124)- and des-(119-124)-RNase (see the previous reports). This may be stated as follows.

Nuclease-(1-126) is flexible although it contains 85% of the amino acid sequence of nuclease from the NH_2-terminus. Neither des-(121-124)-RNase nor des-(119-124)-RNase can yield the native form in large population after reduction and oxidation. This is not interpreted as showing that Nuclease-(1-126) and the reduced RNase derivatives cannot fold to the native three-dimensional structure but as showing that the specific force stabilizing the native three-dimensional structure is not operational in the "native" form of these derivatives.

Examination of the effect of ligands binding on the rate of unfolding of Nuclease-T by exchange between the free fragment and that fragment incorporated in Nuclease-T indicates that in the three-dimensional structure interatomic interactions removed from each other in space strengthen each other, lowering the conformational energy without a large change in conformation. We hypothetically identify the specific force operating only in the native three-dimensional structure as this self-reinforcing three-dimensional linkage of interatomic interactions, although the mechanism of this linkage is unknown. This non-additivity of interaction energy is in contrast to the various forms of interaction energy considered in the conventional calculation of the conformational energy as additively contributing to the total conformational energy.

Methods Employed and Major Findings: As reported last year, the system of Nuclease-(1-126) and Nuclease-T-(50-149) which forms simultaneously two alternative, enzymically active complementing structures, type I and II has been used as a test object of the hypothesis. The study is now complete and is summarized as follows.

The ratio of type I to type II complex formed from the two fragments was determined as a function of time, temperature and the presence or absence of ligands on the basis of the quantities of their derived complexes obtained by limited digestion with trypsin. The ratio of type I to type II complex formed in 2 min after mixing was approximately 0.3 and appears to be independent of temperature and the presence or absence of ligands. The equilibrium between type I and II complexes is attained through unfolding and folding. The ratios of type I to type II complex at the apparent equilibrium state of the system at 6 and 23° were approximately 1.1 and 2.4, respectively. The observations indicate that the rate of unfolding of type II complex is greater than that of type I complex at 6° and increases more than that of type I complex with increasing temperature. Thus, the change of the complementing structure from type I to type II causes a decrease in the activation

free energy, an increase in the activation enthalpy and thereby an increase in activation entropy of unfolding.

Since the unfolded states with which type I and II complexes are in equilibrium are the same, the distribution of the populations of type I and II complexes at the equilibrium state will be determined on the basis of the respective decreases in Gibbs standard free energy from the unfolded state to type I and II complexes. On this basis type I complex has a lower energy by $\Delta G°$=-0.05 and -0.51 kcal mol^{-1} at 6 and 23°, respectively than type II complex. Nevertheless, at the initial complementation the population of type i complex formed is approximately one-third that of type II complex at both 6 and 23°. That is, the probability (rate) of folding is not related to the decrease in energy from the unfolded to the folded state. Using van'T Hoff's equation ΔH=7.5 kcal mol^{-1} and then $\Delta S°$=27 cal $degree^{-1}$ mol^{-1} from type II to type I complex.

The results are consistent with the predictions from the hypothesis that the main decrease in the conformational energy would occur by operation of the self-reinforcing three-dimensional linkage of interatomic interactions after the disordered nuclease folds to a conformation (a transitional state) similar to the native conformation. Thereby the rate of folding would not be related to the decrease in the conformational energy and would be controlled by a statistical factor.

Nuclease-(1-126): As described above, we assume that the conformational properties of Nuclease-(1-126) resemble those of the disordered nuclease under physiological conditions with which native nuclease is in equilibrium. The fragment has been found to have a low level of enzymic activity. Recently D. Sachs, A.N. Schechter, A. Eastlake and C.B. Anfinsen have demonstrated that this enzymic activity is intrinsic. Diana Parker has now contributed to the clarification of enzymic properties of Nuclease-(1-126) by determining the maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m) together with V_{max}, K_m and K_I (dissociation constant of deoxythymidine-3',5'-diphosphate, pdTp) of nuclease, Nuclease-T (Nulcease-T-(6-48) plus Nuclease-T-(50-149)) and type II complex (Nuclease-(1-126) plus Nuclease-(99-149)). The values obtained are as follows. With heat denatured DNA as a substrate-Nuclease: K_m, 7.0 µg/ml V_{max}, 2.7×10^{4} units/µmole; K_I, 1.0×10^{-6} M. Nuclease-T: K_m, 20.0 µl/ml; V_{max}, 2.0×10^{3} units/µmole; K_I, 1.9×10^{-6} M. Type II complex: K_m, 48.2 µg/ml; V_{max}, 2.3×10^{3} units/µmole. Nuclease-(1-126): K_m, 26.2 µg/ml; V_{max}, 25.8 units/µmole. With deoxythymidine-3'-phosphate 5'-p-nitrophenylphosphate (nitrophenyl-pdTp) as a substrate Nuclease: K_m, 1.7×10^{-5} M; V_{max}, 13.3 mole/min/mole; K_I, 1.4×10^{-7} M. Nuclease-T: K_m, 4.6×10^{-5} M; V_{max}, 0.32 mole/min/mole; K_I, 3.3×10^{-6} M. Type II complex: K_m, 6.4×10^{-5} M; V_{max}, 0.11 mole/min/mole; K_I, 7.4×10^{-6} M. Nuclease-(1-126): K_m, 6.8×10^{-4} M; V_{max}, 0.012 mole/min/mole.

Thus, the affinity toward the substrates of Nuclease-(1-126) is lower only by one order of magnitude than that of nuclease. However, the activity of Nuclease-(1-126), even saturated with the substrates, is only one thousandth that of nuclease, in spite of the fact that Nuclease-(1-126) contains all the groups forming the active site of nuclease.

A precursor of nuclease: As described last year, ion exchange chromatographic subfractions have been found to contain a nuclease precursor in both

V8 and Foggi strains. The contribution by Austine Davis has now enabled this species to be identified on the basis of the amino acid sequence. The precursor of Foggi strain contains the 18 extra residues of the sequence Ser-Glx-Thr-Asp-Asx-Gly-Val-Asx-Arg-Ser-Gly-Ser-Glu-Asp-Pro-Thr-Val-Tyr bonded by a peptide linkage to the NH_2-terminus of nuclease. A variation of the precursor appears to occur depending on the presence and absence of amide groups at the residues indicated by Asx and Glx. The extra amino acid sequence (numbered as P1 to P18 from the NH_2-terminus) and the portion of residues 1 to 5 of the precursor can be specifically digested away with trypsin in the presence of ligands (pdTp and Ca^{2+}). The fragments thus removed have been used in determining the sequence. The observations indicate that the extra amino acid sequence is flexible similarly to the portion of residues 1 to 5 of nuclease. Staphylococcal protease (extracellular, G.R. Drapeau, Y. Boily and J. Houmard J. Biol. Chem. 247, 6720 (1972)) is also found to cleave the bond between residue P18 and residue 1 in the presence of ligands forming nuclease. The precursor does not contain sugar moieties in either V8 or Foggi strain.

X-ray crystallographic study of Nuclease-T: Synthesis of Nuclease-T-(6-48) is being done by Dr. Carlo DiBello, University of Padova, Italy in collaboration for the project of crystallization of semisynthetic Nuclease-T (the previous project No. Z01AM25,010-02LCB). The objective of this particular approach to the study of interatomic interactions is that measurements of the interaction energy maintaining the three-dimensional structure by the method developed in our laboratory be combined with the X-ray crystallographic studies of semisynthetic Nuclease-T with substituted residues in order to understand quantitatively the contribution of residues to the interaction energy in proteins.

Photographic measurements of X-ray diffraction became possible in this section a few months ago. The X-ray crystallographic study of the structure of Nuclease-T has now begun with the collaboration of Dr. Gerson Cohen, the Section on Molecular Structure, Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases.

Digestion of Nuclease-T-(6-48) with staphylococcal protease appears to yield Nuclease-(6-43) which combines with Nuclease-T-(50-149) to form an enzymically inactive complex.

Significance to Biomedical Research and the Program of the Institute: Interatomic interactions in proteins which are vital for maintaining the three-dimensional structures are involved in the mechanism of enzymic activity and are related to both protein-ligand and protein-protein interactions and are not completely understood. The studies of the complementing fragments of staphylococcal nuclease have given insight into the principles underlying these interatomic interactions. Specifically, we have developed the hypothetical concept of the self-reinforcing three-dimensional linkage of interatomic interactions which would provide the stabilizing energy of the three-dimensional structures and a way to transmit the effect of a change in interatomic interactions at one site of proteins through the three-dimensional structures. The physical reality of this hypothetical linkage of interatomic interactions is unknown. An effort will be made to understand the physical reality of

this linkage in future studies.

Proposed Course:

1) Determination of the atomic coordinates of Nuclease-T.
2) Crystallization and X-ray diffraction studies of semisynthetic Nuclease-T' with substituted residues.
3) Crystallization and X-ray diffraction studies of the inactive Nuclease-T analogue containing Nuclease-(6-43) bound with nitrophenyl-pdTp and other substrates.
4) Crystallization and X-ray diffraction studies of type II complex.
5) Thermodynamic studies of interaction of Nuclease-(1-126) with ligands.
6) Studies of equilibration of type I and type II complexes containing substituted residues.
7) Measurements of the energy barrier of unfolding of semisynthetic Nuclease-T with substituted residues.
8) NMR studies of nuclease fragments.

Publication:

Taniuchi, H., Parker, D.S. and Bohnert, J.L. A Study of Equilibration of the System Involving Two Alternative, Enzymically Active Complementing Structures Simultaneously Formed From Two Overlapping Fragments of Staphylococcal Nuclease. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,009-02 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies of the Mechanism of Native Pairing of Half-cystine in Proteins Containing Disulfide Bonds

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

PI:	S.A. Acharya	Visiting Associate	LCB NIAMDD
	H. Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Conformation Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

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

The material obtained from reduced hen egg white lysozyme after complete air oxidation were fractionated by gel filtration to the enzymically active, native species and to the enzymically inactive form which eluted at an elution volume smaller than that of the native species but greater than that expected for a dimeric form of lysozyme. The yield of the inactive form increased up to 100% when the oxidation of reduced lysozyme was accelerated using cupric ion. This inactive form, shown to contain incorrect disulfide bonds, can be quantitatively renatured by sulfhydryl-disulfide interchange. In the early phase of oxidation of reduced lysozyme three partially oxidized, active species were trapped by alkylation with ^{14}C-iodoacetate. Isolation and characterization of the radioactive tryptic peptides from each of the 3 active forms, permitted the identification of Cys-6 and Cys-127, Cys-76 and Cys-94, and Cys-80 respectively as the free sulfhydryl groups in the three incompletely oxidized species. Thus, these active species appear to contain three native disulfide bonds and one open disulfide bond. Trapping, by alkylation, the active species containing sulfhydryl groups formed during the renaturation by sulfhydryl-disulfide interchange, of the inactive species and examination of the radioactive tryptic peptides have suggested that the active species containing three native disulfide bonds and one open disulfide bond can be formed in all possible cases.

Project Description:

Methods Employed and Major Findings:

1) Separation of enzymically inactive forms of lysozymes on columns of Biogel P-30 and their characterization. Previous studies have shown that on the renaturation of reduced lysozyme two populations of molecular species are formed which could be separated by gel filtration on Biogel P-30; an enzymatically active species eluting at the position of native lysozyme and an enzymically inactive species eluting at a position slightly earlier to that of native protein, but very much after the position expected for a dimeric species of lysozyme. The molecular species generated by the oxidation of reduced lysozyme in 6 M guanidine hydrochloride containing apparently random disulfide bonds also eluted at the position of the enzymically inactive species. The material obtained by the randomization of the 'correct' disulfide pairing of the native lysozyme using catalytic amounts of β-mercaptoethanol in 6 M guanidine hydrochloride or 8 M urea also eluted in the same position. All these enzymically inactive species can be renatured to the active species by thiol-disulfide interchange catalyzed by β-mercaptoethanol.

Evidence for the presence of incorrectly paired disulfide bonds in these inactive species, designated higher hydrodynamic volume species, has been obtained by CNBr cleavage. The inactive species obtained by fast oxidation, showed that 25% of its population contained non-native disulfide bonds between cystine residues 115 and 127. If the pairing of half cystine residues is completely random, the material should have contained only 14.3% of such population. On the other hand, the inactive species generated by the randomization of the native protein, contained the non-native 115-127 disulfide bond in at least 45% of their population. The material generated by oxidation of reduced lysozyme in 6 M guanidine hydrochloride was found to contain such non-native disulfide bonds in at least 70% of their population. Still all these species eluted at the same position from the Biogel columns distinct from that of native protein, and were renatured to the native in almost quantitative yields. This suggests that the formation of native or native-like lower hydrodynamic volume species is an all or none phenomenon. If the increase in the elution volume of the reduced lysozyme on oxidation is a linear function of the number of the native disulfide bonds, one would not expect such a clear cut separation of the native form from the non-native species on gel filtration. This may be considered as a reflection of the distinct state of intratomic interactions in the native three-dimensional structure.

Viscosity measurements and fluorescent studies have been carried out with the higher hydrodynamic volume species in an attempt to obtain information about the molecular conformation of these derivatives. The intrinsic viscosity of higher hydrodynamic volume form (5 ml per g) is nearly twice that of native lysozyme indicating that the inactive molecule has an enlarged molecular domain. Reduced lysozyme and reduced and carboxymethylated lysozyme in 0.1 M acetic acid gave an intrinsic viscosity of 12.4 ml per g, a value lower than that found in 6 M guanidine hydrochloride for the same material (16.4 ml per g). The presence of 6 M guanidine hydrochloride increased the intrinsic viscosity of the higher hydrodynamic volume forms as well as that of the native lysozyme to 9.4 ml per g, pointing out that the molecular domain of the higher hydrodynamic volume species in the absence of the dena-

turant is smaller than that in the presence of denaturant. A comparison of fluorescence emission spectrum and wavelength of emission maximum due to the tryptophan residues of lysozyme suggests that the environment of tryptophan residues of native lysozyme at the pH 4.8 is less polar than those of reduced lysozyme and the higher hydrodynamic volume form, and that in the presence of 6 M guanidine hydrochloride the polarity of the environment of tryptophan residues of all these sample increases. These results are consistent with a disordered conformation for these derivatives, although the precise state of disordered conformation appears to be different between these derivatives.

2) Enzymically active intermediates formed during reoxidation. Earlier studies have shown that three enzymatically active lower hydrodynamic volume species, trapped with iodoacetate, are formed during the reoxidation of reduced lysozyme. Two of these derivatives had two moles of S-carboxymethylcystines, whereas the other one had only one mol of S-carboxymethylcysteines. Previously one of the dicarboxymethylated derivatives has been identified as having the carboxymethyl groups on cysteines 6 and 127.

The second dicarboxymethyl derivative of lysozyme had both the carboxyl groups in the tryptic fragment T_{11} (residues 74 to 96) having half cystine residues at positions 76, 80 and 94. Characterization of the peptic fragments of this T_{11} showed that one of the carboxymethyl group is at position 94, the other one in the fragment 74 to 92, probably at 76, since these two residues are paired in the native molecule. The monocarboxymethylated derivative of lysozyme appears to have its half cystine residue 80 alkylated. Presumably, the half cystine 64 is free in this derivative. These results suggest that interatomic interactions stabilizing the native three-dimensional structure of lysozyme are operating even when one of the disulfide bonds between residues 6 and 127, 76 and 94, and 94 and 80 are not formed.

3) Rearrangement of higher hydrodynamic volume forms. It has been shown that the formation of native lysozyme during reoxidation of reduced lysozyme can not be formed without sulfhydryl-disulfide interchange. The rearrangement of the higher hydrodynamic volume form of lysozyme to its native forms in the presence of β-mercaptoethanol occurs at a much faster rate than the renaturation of reduced lysozyme. Therefore it is unlikely that in the renaturation of the higher hydrodynamic volume, the protein is converted back to the reduced state, and the disulfide bonds are formed one after another in a sequential manner. It is more likely that one or two disulfide bonds in the non-native molecule could be opened by intermolecular sulfhydryl disulfide interchange with β-mercaptoethanol and then converted by intramolecular rearrangement to the native form. Then the question would arise as to how many native disulfide bonds have to be formed before the rearrangement of the disulfide bonds by sulfhydryl disulfide interchange becomes effective. In an attempt to answer these questions, the rearrangement of higher hydrodynamic volume species has been undertaken.

The higher hydrodynamic volume species formed on randomizing the disulfide bonds of native lysozyme in 8 M urea using β-mercaptoethanol are used in the present study. These were rearranged with β-mercaptoethanol at pH 8.0, 37° and reaction mixture was alkylated with ^{14}C-labelled iodoacetic. The lower and higher hydrodynamic volume species were isolated by chromatography on Biogel P-30. The higher hydrodynamic volume species thus isolated had about 4 to 4.3 mol of S-carboxymethylcystine. The lower hydrodynamic volume

species appeared to contain about 1 mol of S-carboxymethylcystine (after applying correction for the presence of fully renatured lysozyme). Apparently the lower hydrodynamic volume structures formed have only one open disulfide bonds in them. A chromatographic analysis of the tryptic peptides showed that any one of four disulfide bonds of native lysozyme is dispensible for the formation of lower hydrodynamic volume structure of lysozyme.

4) Reduction and reoxidation of the derivative of lysozyme containing carboxymethylated cystine residues at 6 and 127. In an attempt to study whether the necessary information present in the amino acid sequence of reduced lysozyme for the formation of native three-dimensional structure is also present in its derivative in which the cystine 6 and 127 are alkylated, its reduction and reoxidation has been followed. This reduced protein was found to be capable of forming the lower hydrodynamic volume structure in 50 to 60% yield. The renatured material was also found to be identical with the starting material in its chromatographic behavior on IRC-50 columns and enzymatic activity. It was also shown that this derivative could be randomized in urea using β-mercaptoethanol, and then renatured by sulfhydryl-disulfide interchange. However the yield of the native hydrodynamic volume material was low in this system (about 18 to 25%). This renaturation study after reduction, clearly shows that this particular derivative is of lowest conformational energy. Preliminary studies with the other dicarboxymethylated derivative (half cystines 76 and 94 alkylated) also show that this derivative also could be reduced and reoxidized to the lower hydrodynamic volume structure.

Significance to Biomedical Research: The results of the present investigation suggest that the interatomic interactions responsible for the formation of, and the stabilization of the native structure operate even before all the four native disulfide bonds of lysozyme are formed. Even in the absence of any one of the four native disulfide bonds of lysozyme, these interatomic interactions are sufficiently strong to constrain the lower hydrodynamic volume structure, characteristic of the native protein. The information of this kind will contribute to a more precise understanding of the molecular recognition processes, the fundamental principle of life processes.

Proposed Course of Research:

1) To establish the monomeric nature of the higher hydrodynamic volume species and to determine its stokes radius.

2) Isolation of all the four isomeric forms of lysozymes with one open disulfide bond.

3) Characterization of the native pairing of half-cystine residue bonds in these derivatives.

4) A study of dispensability of these disulfide bonds in the reduction and reoxidation process.

5). Crystallization and X-ray diffraction studies of the above derivatives.

6) Look for a possible derivative of lysozyme with lower hydrodynamic volume structure containing only two of the four native disulfide bonds.

7) Carboxypeptidase and aminopeptidase digestion of the derivative carboxymethylated at cystine residues 6 and 127 in an attempt to get a complementing system to explore further the folding process of reduced lysozyme.

Publication:

Acharya, S.A. and Taniuchi, H.: A Study of Renaturation of Reduced Hen Egg White Lysozyme: Enzymically Active Intermediates Formed During Oxidation of the Reduced Protein. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 25,010-03

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies on the Relationship Between the Primary and Tertiary Structure of Proteins: The Crystallization and X-ray Diffraction Study of Semisynthetic Nuclease T'

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

Principal Investigator:

Hiroshi Taniuchi — Chief, Sec. on Protein Conformation — LCB NIAMDD

COOPERATING UNITS (if any)

Section on Molecular Structure, Lab. of Molecular Biology, NIAMDD, Institute of General Chemistry, University of Padova, Padova, Italy (Dr.Carlo DiBello)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Protein Conformation Section

INSTITUTE AND LOCATION

NIAMDD:NIH; Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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

1) Crystallization and X-ray diffraction study of semisynthetic nuclease T'.

2) Crystallization, X-ray diffraction and thermodynamic studies on semisynthetic nuclease T' analogues composed of selectively modified synthetic nuclease-T-(6-48) with nuclease-T-(49-149).

3) Systematic study of synthetic methods intended to improve the overall yield of peptides required for the proposed studies on the semisynthetic nuclease T' system.

4) Total synthesis, crystallization and X-ray diffraction study of nuclease T'.

This project is now consolidated with other work being done in the Section and is covered in Project #ZO1 AM 25,008-13 LCB

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,011-02 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Protein Unfolding by Fragment Exchange with a System Derived from Cytochrome c

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

PI:	R.R. Hantgan	Staff Fellow	LCB NIAMDD
	H. Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Conformation Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

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

The complex formed from the ferric fragment (1-65)H of cytochrome c and apo-cytochrome c was treated by limited digestion with trypsin in order to remove the redundant residues. The components of the derived complex were separated by ion exchange chromatography in 8 M urea. The three major non-heme and two major heme fragments were tentatively identified as (40-104), (54-104), (39-104),(1-53)H and (1-55)H. Measurements of the fluorescence quenching of tryptophan 59 of the non-heme peptides by the ferric peptides indicated that a 1:1 complex is formed in all 6 cases with a dissociation constant of less than 3×10^{-7} M. The complex (40-104):(1-53)H, which is obtained in the largest amount, has a K_{diss} $< 3 \times 10^{-8}$ M and approximately 30% of the activity of native cytochrome c when reduced by lactate dehydrogenase from Baker's yeast. Thus cytochrome c can be cleaved at specific sites between residues 39 and 55 and reconstituted as a non-covalent complement with physical and biological properties similar to the parent molecule. This region containing the major sites of permissible trypsin cleavage is the same as that found by R.E. Dickerson et al. to have been excised in Pseudomonas cytochrome c_{551} during the course of evolution.

PHS-6040
(12-75)

Project Description:

Objective: Estimation of the stabilization energy maintaining the structure of cytochrome c; finding a relationship between the stabilization energy and the atomic co-ordinates of the protein.

Methods Employed and Major Findings: It has been shown (Fisher et al., J.B.C., 248: 3188, 1973) that the cyanogen bromide ferrous heme fragment (residues 1 to 65) of cytochrome c (Corradin and Harbury, P.N.A.S., 68: 3036, 1971) can complement with apo cytochrome c. The previous report (see NIAMDD-LCB-12, 1974-75) described the purification of cytochrome c, the preparation of the apo protein and the preparation of the (1-65) heme fragment. The change in the Soret spectrum on mixing equimolar quantities of the apo protein and heme fragment indicated an ordered complex could be formed from the presumably structureless polypeptides. Preliminary results, based on peptide maps and spectral characteristics of the complex indicated a limited trypsin digestion could remove redundant residues without disturbing the native-like character of this non-covalently associating system.

Further proof of the formation of structure came from measurements of the fluorescence of tryptophan residue 59 of cytochrome c. In the native protein, this tryptophan does not fluoresce as it is quenched by energy transfer to the proximate heme group (Vanderkooi and Ericinaka, E.J.B. 60: 199, 1975). Upon denaturation, substantial tryptophan fluorescence is regained (Tsong, J.B.C. 249: 1988, 1974). Similarly, denaturation of the complex (1-65)H:(1-104) by 6 M guanidine hydrochloride at pH 7 results in a large increase in fluorescence at 350 nm.

Also, the fluorescence quantum yield of native cytochrome c was shown to increase during the course of a trypsin digestion of the ferric protein. As the ferrous protein is resistant to such proteolysis, the complex (1-65)H:(1-104) was kept reduced under anaerobic conditions, trypsin was added, and the fluorescence yield followed as a function of time of digestion. These experiments indicated a limited trypsin cleavage could remove redundant portions of the complex (as verified by peptide maps) without disturbing the native-like structure of the subsequent complementing system.

With conditions clearly defined, a preparation involving 100 mg of (1-65)H:(1-104) was carried out. The trypsin surviving complements were separated from the removed peptides by gel filtration. Attempts to separate the complementing fragments by gel filtration in denaturing solvents (0.1 M and 30% acetic acid) gave inadequate resolution. Also an affinity chromatography column made by coupling the (1-65)H peptide to CNBr-Sepharose 4B was prepared. This column could bind (1-104) from a solution but could not compete effectively for (1-104) when complexed in solution with (1-65)H. Thus it was not expected to be able to separate the trypsin surviving fragment.

Ion exchange chromatography in 8 M urea proved most effective here, and the initial experiment revealed three major non-heme peptides, and two heme peptides. These were tentatively identified, by amino acid analysis and peptide maps, as (40-104), (54-104), (39-104), (1-53)H and (1-55)H. Measurements of the fluorescence quenching of tryptophan 59 of the non-heme peptides by the ferric peptides indicated a 1:1 complex is formed in all six cases

with a dissociation constant of $<3 \times 10^{-7}$ M. The complex obtained in the largest yield, (1-53)H:(40-104) had a K_{diss} $<3 \times 10^{-8}$ M and approximately 30% of the activity of native cytochrome c when reduced by lactate dehydrogenase from Baker's yeast.

This preparation was repeated using 250 mg of complex; a similar elution profile resulted from the ion exchange in 8 M urea. The non-heme peptides were identified by amino acid analysis, peptide maps, and stepwise Edman degradation. Removal of 3 or 4 amino acids from their N-termini unambiguously identified the three major non-heme components as (1) a mixture of (40-104) and (56-104), (2) (54-104) and (3) (39-104). Peak (1) was further separated into its two components by gel filtration in 0.2 M acetic acid. Peaks (2) and (3) were greater than 90% pure by the criterion of amino acid analysis, peptide maps and Edman degradation.

The major heme peptide was tentatively shown to be (1-53)H by amino acid analysis and peptide maps. The smaller, more retarded peak of Soret absorbance appeared to have two components, possibly (1-55)H and (1-60)H. To verify the sequence of the major peak, a derivative was prepared in which all lysine residues were trifluoroacetylated. A trypsin digestion can cleave only at residue 38, arginine (Farger and Harbury, Biochemistry 4: 2541, 1965). The peptide (38-53) can be separated from the heme piece by gel filtration and identified by amino acid analysis. Here fragment (1-55)H will yield a peptide with 3 lysines and 3 aspartic acid groups compared to the 2 residues of each for the (1-53)H fragment. The accuracy of the analyzer should be sufficient to distinguish these possibilities.

To determine the activity of these complexes in the classical Keilin-Hartree assay (Ferguson-Miller, Brautigan and Margoliash, J.B.C. 251: 1104, 1976), the components of a polorographic device to measure oxygen consumption have been assembled into a functioning instrument. The equipment and materials necessary to isolate a cytochrome oxidase-reductase system from beef heart are being acquired at this time.

Significance to Biomedical Research: The complementing peptides of cytochrome c are being prepared as part of a fragment exchange system similar, in principle, to that devised in this laboratory with staphylococcal nuclease. The method permits measurement of protein unfolding rates under physiological as well as denaturing conditions. A determination of these exchange rates as a function of temperature will allow the estimation of the energy maintaining the unique tertiary structure of a protein. Then specific modifications in the amino acid sequence of one of the peptides can be introduced, either by chemical synthesis or a careful choice of cytochrome c from a different species. In this manner the effect of modifications on the primary structure can be related to the energy stabilizing the tertiary structure.

Cytochrome c is interesting for its importance to metabolism, its well defined structure, and the many permissible alterations in its sequence which still preserve its structural integrity. In this context, it is interesting to note that the structure of Pseudomonas cytochrome C_{551} has been determined; in this molecule residues 38-57 have been excised during the course of evolution and the remainder of the polypeptide chain rearranged to preserve the cytochrome c structure (Dickerson et al., J. Mol. Biol. 100: 473, 1976).

This 38-57 region contains all the major sites of permissible trypsin cleavage of the parent complement in this study. Thus the regions of cleavage, both *in vivo* and *in vitro* appear to be *not* chosen randomly, but selected in such a way as to preserve the function of this molecule.

Proposed Course of Research:

1) Determine the activity of the complements in both cytochrome oxidase and reductase assays.

2) Determine $K_{Dissociation}$ values for the complements.

3) Measure spectral and fluorescent properties of the complex to establish its native-like character.

4) Crystallization and X-ray studies of the complex.

5) All this with the eventual goal of measuring fragment exchange rates with these cytochrome *c* complements.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,013-05 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Monospecific Antibodies as Probes of Hemoglobin Structure and Function

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

PI:	J. Dean	Research Associate	LCB NIAMDD
	A. Eastlake	Chemist	LCB NIAMDD
	A.N. Schechter	Chief, Sec. on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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

Using non-precipitating monospecific antibodies as probes of conformation, we are studying structural differences of hemoglobin in solution. Hemoglobin A_o and A_2 have been isolated, purified and carbamoylated with $KC^{14}NO$. Two specific subpopulations of anti-hemoglobin antibodies have been isolated by affinity chromatography using synthetic peptides corresponding to $\alpha(129-141)$ and $\delta(1-13)$. The first subpopulation will be used to study conformational changes at the carboxyterminus of the α chain during oxygenation based on the difference in the binding affinity between C^{14} oxy and C^{14} deoxy hemoglobin. Differences will be used to examine mutant and truncated hemoglobins so as to better understand the structural changes occurring during the dynamic translation from the T (deoxy) state to the R (oxy) state. The second subpopulation will be used to measure hemoglobin A_2 levels in serum and to differentiate A_2 from other normal and mutant hemoglobins.

PHS-6040

Project Description:

Objectives: For the last 4-5 years, this laboratory has been interested in the use of monospecific antibodies as probes to study conformational changes of proteins in solution. Initially work was begun with antibodies specific for staphylococcal nuclease fragments and later with antibodies directed against the amino terminal and of the β chain of sickle hemoglobin. More recently we have directed our attention towards two other projects using antibody subpopulations as probes: 1) The detection of confomational differences of normal hemoglobin A_o as it changes from the T state (deoxy) to the R state (oxy) upon oxygenation. 2) The quantitative measurement of Hemoglobin A_2 ($\alpha_2\delta_2$), a normally occurring hemoglobin, by means of a radioimmunoassay using antibodies specific for a 13 residue peptide contained in the δ chain.

Methods and Major Results: Hemoglobin A_o and A_2 have been chromatographically purified by DEAE Sephadex and isotopically carbamoylated with $KC^{14}NO$. Studies are underway to demonstrate that hemoglobin so treated is still functionally active.

Whole antisera from sheep immunized with A_o have been obtained and their ability to discriminate between the R (oxy) and T (deoxy) states is now being analyzed. Using the Corley modification of Merrifield solid phase synthesis, the carboxyterminus of the α chain (α 129-141) has been synthesized and utilized in affinity chromatography to isolate from the whole antisera a subpopulation of non-precipitating antibodies specific to α(129-141).

A precipitating second antibody (burro anti-sheep IgG) is used to separate C^{14} oxyhemoglobin-anti α(129-141) antibody complexes from uncomplexed labeled hemoglobin and the slope of a Scatchard analysis is used to determine the binding constant of these antibodies to oxyhemoglobin. A similar analysis is used to calculate the binding constant of the α(129-141) antibodies to C^{14} deoxyhemoglobin. However, in the dilute hemoglobin solution that we are using great care must be taken to prevent the oxidation of ferrous hemoglobin to the ferric state (methemoglobin) at low partial pressures of oxygen that are used during the deoxygenation process. To prevent this we have stored the C^{14} hemoglobin in the carbonmonoxy liganded form and, then, just prior to use, oxidized it to the ferric state *in vacuo* to release the CO, reduced it under anerobic conditions using pig heart diaphorase and thus, obtained deoxy ferrous hemoglobin. The binding assay is then performed in the NIH Anerobic Chamber using small amounts of sodium dithionite as an oxygen scavenger.

An analogous strategy is being used to obtain monospecific antibodies to hemoglobin A_2. An external portion of the delta chain (δ 1-13) has been synthesized, attached to an affinity column and an antibody subpopulation obtained. These antibodies will be used in a radioimmunoassay to quantitate levels of A_2 and examine the extent of cross reactivity with other normal and mutant hemoglobins.

Significance: Elegant studies by Perutz and his colleagues in the 1960's have shown significant tertiary and quarternary changes in the conformation of hemoglobin upon oxygenation. We hope that an assay using monospecific antibodies to oxyhemoglobin in solution will complement these crystallographic

studies and give further information about the dynamics of this conformational shift. A variety of perturbations - breaking salt bridges, examining truncated and mutant hemoglobin - may give us further insight into the molecular structural changes involved in this important but poorly understood transition.

Antibodies specific to A_2 will have direct clinical application in measuring levels of A_2 in the fetus and in the adult, as well as, distinguishing A_2 hemoglobin from others (such as Hemoglobin C) which have a similar electrophoretic mobility.

Proposed Course: Continued development of the above approaches to study conformation differences among normal and mutant hemoglobins.

Publications:

Curd, J.G., Ludwig, D. and Schechter, A.N.: Antibodies to an Amino Terminal Fragment of β^S Globin. I. Preparation and Radioimmunoassay. J. Biol. Chem. 251, 1283-1289 (1976).

Curd, J.G., Young, N. and Schechter, A.N.: Antibodies to an Amino Terminal Fragment of β^S Globin. II. Specificity and Isolation of Antibodies for the Sickle Mutation. J. Biol. Chem. 251, 1290-1295 (1976).

Schechter, A.N.: The Conformation of Peptides and Proteins in Solution: Immunochemical Studies. Clark, J.H., Klee, W., Levitski, A. and Wolff, J. (Eds.): Hormone and Antihormone Action at the Target Cell, Dahlem Konferenzen, Berlin, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 25,014-02 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Functional Properties of Immune Response Gene Regulation of Antibody Response to Nuclease

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

Principal Investigators:

Jay A. Berzofsky	Research Associate	LCB NIAMDD
Alan N. Schechter	Chief, Sec. on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)
Immunology Branch, NCI (David H. Sachs and Gene M. Shearer)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD:NIH; Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

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

The ability to raise an immune response to specific antigens has recently been shown to be under genetic control, linked to the major histocompatibility complex (MHC) in several species studied. In the mouse, a number of synthetic antigens have been found subject to this H-2 linked control, but only a few natural globular proteins have been studied as antigens, among these, staphylococcal nuclease. We have now tried to delineate the individual antigenic determinants on this protein which are responsible for this genetic control of the immune response in inbred strains of mice, in order to better understand the mechanisms involved. We have found that a single peptide fragment of the protein appears to be under the same control as the whole molecule. Furthermore when antibodies made to the whole nuclease molecule are studied for their specificity, we have found that the H-2 linked immune response genes can control the response to individual determinants on the same molecule independently of one another, and therefore may be involved in the selection of specific B lymphocytes rather than only T cells.

PHS-6040
(12-75)

Project Description:

Objectives: It has previously been shown that immune response genes (Ir genes) which map within the major histocompatibility comples (H-2) of mice determine the ability of various inbred strains of mice to response to diverse antigens with antibody production and/or cellular immunity. These are apparently a separate level of regulator genes, which have the ability to recognize and distinguish a broad range of immunogenic determinants. In synthetic polypeptide antigen studies these determinants have generally been identified with backbone or "carrier" determinants. However, natural protein antigens have not been widely studied, and it is not clear what part of the molecule might serve this "carrier" function, or how it might be recognized. One objective of this study is to understand these mechanism. Previous investigations have shown that the antibody response to staphylococcal nuclease in mice is under H-2 linked Ir gene control. In order to understand the mechanisms of Ir gene control of the response to natural globular protein antigens, we have undertaken to delineate the response to nuclease by a study of the course of the response with time and by using peptide fragments representing subregions of the molecule in five inbred strains of mice.

Major Results and Methods Employed:

1. Using an assay for antibodies to whole nuclease by inhibition of enzymatic activity, we have found that the strains A/J and B10·A, which share the same high responder H-2^a haplotype but differ at other genetic loci, have consistently different levels of anti-nuclease response even with multiple boosting. This result implies non-H-2-linked control of the magnitude of the response as well as the H-2 linked control.

2. The B10 strain (H-2^b), a low responder in the initial response, eventually produces antibody levels comparable to the high responder B10·A congenic strain after several boosts, when inhibition of whole nuclease is the assay.

3. Using newly developed radioimmunoassays for antibodies binding to ^{14}C-labeled fragments of nuclease corresponding to residues 99-149 and 1-126 (prepared as described in the previous report), it was found that the concentration of antibodies made to whole nuclease which bound to the 1-126 fragment was approximately the same in the two congenic strains B10 and B10·A. In contrast, a markedly lower concentration of the antibodies bound to the 99-149 fragment in the B10 than in the B10·A strain. Therefore, since the strains differ only at H-2, an H-2 linked Ir gene can control the response to individual determinants on the same antigen molecule independently of one another, contrary to expectations.

This result also explains the delayed response of B10 mice compared to B10·A when binding to whole nuclease was measured, since the B10 mice never produced as much antibody to the 99-149 region, but presumably were making antibodies to weaker determinants in other regions of nuclease after multiple boosts.

4. The high responder A/J and SJL mice were also found to make a significant response to the 99-149 region of nuclease when immunized with the whole molecule.

5. When the individual fragments themselves were used as immunogens in five strains of mice (3 high and 2 low responders), and sera from individual mice tested individually using groups of 8-12 mice per strain per immunogen over 3 immunizations, it was found that response to the 99-149 fragment followed the same pattern as that to whole nuclease. However, the B10, which was a low responder to nuclease and showed increased response with boosting, was a nonresponder to the 99-149 fragment, even after 3 immunizations. In contrast, all of the strains (except the other low responder, DBA/1) responded to the 1-126 fragment. A third fragment, 6-48, used as a control for its similarity to 99-149 in size and charge, was a poor immunogen in all 5 strains studied.

Thus, the response to one peptide fragment of nuclease appears to be under the same genetic control as that to whole nuclease, despite the largely random conformation of the peptide.

This work has been presented at the 1976 meeting of the American Association of Immunologists (Fed. Proc. 35: 627, 1976), and several manuscripts are in preparation for publication.

Signifance to Biomedical Research: The mechanism of the genetic control of the immune response is important in understanding the steps leading from immunization with an antigen to the final production of antibodies. It is also important in understanding the diseases whose pathogenesis is partially immunological--such as the autoimmune diseases and immune deficiency states, including the specific immune deficiency seen in cancer. Our results have the following specific implications about this genetic control: 1) The additional non-H-2 linked control of the antibody response suggests other independent mechanisms of control which may be studied. 2) The finding of a fragment of nuclease which may be under the same control as the whole molecule opens up the possibility of further delimiting the regions which determine the immune response recognition by the use of other natural and synthetic peptides. 3) If the interpretation is correct that H-2 linked genetic control can be the same for a random conformation fragment as for native nuclease, then this suggests either a) that the receptors involved show less conformational specificity than the antibodies ultimately produced (which distinguish sharply between native and random conformations) or b) that the key determinant recognized is short or flexible enough to not differ significantly between the random and native structures. 4) The result that the response to two different determinants on the same molecule can be controlled independently by H-2 linked genes in strains that share the same immunoglobulin structured genes, suggests that H-2 linked Ir gene control is exerted at the level of selection of specific B cells, and raises serious doubt about the popular model which states that the presence of T cells which can recognize a single "carrier" determinant on the molecule is sufficient to allow triggering of B-cell response to any determinant on the same molecule.

Proposed Course of Project: 1) The structure of the determinant responsible for control, on the 99-149 fragment, is to be further delineated chemically. 2) The observation that serum from low responder strains may have a lower level of antigen binding capacity after boosting than before immunization is to be examined for possible anti-idiotype production or induction of suppressor T cells as a mechanism of control of the immune response. 3) Mechanisms to explain the apparent control at the level of B cells specific for individual determinants are to be examined.

Publications:

Sachs, D. H., Berzofsky, J. A., Schechter, A. N., Fathman, C. G., Pisetsky, D. S., and Schwartz, R. H.: The Immune Response to Staphylococcal Nuclease: A Probe of Cellular and Humoral Antigen-Specific Receptors. Cold Spring Harbor Symp. Quant. Biol. 41: (1976) in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,015-02 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Multideterminant Antigens in Radioimmunoassay: Antibodies to Sickle Hemoglobin

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

PI:	J.A. Berzofsky	Research Associate	LCB NIAMDD
	J.G. Curd	Research Associate	LCB NIAMDD
	A.N. Schechter	Chief, Sec. on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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

Analyses of radioimmunoassay curves generally depend on the mass action law equations for simple equilibria between a single antigen and antibody. However, most natural protein antigens have multiple different antigenic determinants, and the antisera made to them are multispecific. The behavior of these systems is too complex to solve in a general analytic formulation, so that only individual cases can be treated numerically by computer. We have now developed a general theory for radioimmunoassay binding curves of multideterminant antigens, using probability theory. The only assumptions are that the determinants be unique and independent in their binding to antibodies. The predictive ability of the theory has been demonstrated for antibodies to subregions of the N-terminal third of the β chain of sickle hemoglobin, studied using antisera fractionated on affinity chromatographic columns of synthetic peptides. One implication is that to obtain quantitative binding parameters, such as affinity constants for multideterminant antigens, one should fractionate the sera to obtain monospecific antibodies.

PHS-6040
(12-75)

Project Description:

Objectives: The commonly used models of antigen-antibody interaction are all based on equilibria between antibodies and relatively simple antigens which can bind only a single antibody molecule at a time. However, most natural protein antigens have multiple determinants, and antisera raised to these have mixtures of antibodies for the different determinants. Yet no theory is available in the literature to describe the behavior of these more complex systems in a general way.

The previous report in this project number described antibodies made to the N-terminal 55-residue peptide from the β chain of Hemoglobin S, which we call $\beta^S(1\text{-}55)$. Radioimmunoassay curves of bound/free vs. total antigen showed unusually steep slopes which were not explained. In the present study we develop a completely general theory of the behavior of multideterminant antigens in radioimmunoassay and then show experimentally that the steep slopes observed in the $\beta^S(1\text{-}55)$ system can be explained by this theory.

Major Results and Methods:

(1) Theoretical: The binding functions of multideterminant antigens may be too complex to solve in a general form by the mass action approach. However, if one assumes that the determinants are unique and independent in interaction with antibodies, then at a given concentration of antiserum and antigen, the probability that no sites will have antibody bound will be the product of the probabilities that each individual site will be unbound. Since one scores an antigen molecule as bound if at least one antibody molecule is attached, the observed fraction bound B for the whole antigen will be one minus the product of the probabilities that each site will be free, i.e., $B=1-\prod_{i=1}^{n}(1-b_i)$, where b_i is the probability that the i^{th} of n total sites will be bound. In a similar fashion, it follows from this result that if R is the observed macroscopic bound/free ratio and r_i is the corresponding ratio for the i^{th} determinant site, then $R+1=\prod_{i=1}^{n}(r_i+1)$. Differentiating this equation with respect to total antigen, T, yields the result $\frac{\partial R}{\partial T}=\sum_{i=1}^{n}\left[\left\{\prod_{j\neq i}(r_j+1)\right\}\frac{\partial r_i}{\partial T}\right]$ so that $\left|\frac{\partial R}{\partial T}\right| > \left|\sum_{i=1}^{n}\frac{\partial r_i}{\partial T}\right|$ unless all the r_i are zero, a trivial case. Thus, as the number of sites n increases, the slope becomes increasingly steeper.

(2) Experimental: In order to demonstrate the applicability of the theory to the $\beta^S(1\text{-}55)$ system, we prepared the smaller peptide $\beta^S(40\text{-}53)$ by the Corley-Sachs-Anfinsen modification of the Merrifield method of solid phase synthesis. This peptide and the previously synthesized $\beta^S(1\text{-}13)$ peptide were each attached to Sepharose by the CNBr method and used to fractionate the antiserum made to the whole $\beta^S(1\text{-}55)$ peptide by affinity chromatography. The resulting subpopulations of antibodies specific for these two regions were called anti-$\beta^S(40\text{-}53)$ and anti-$\beta^S(1\text{-}13)$ respectively.

The radioimmunoassay binding curves for these individual subpopulations of antibodies binding to the whole $\beta^S(1\text{-}55)$ peptide antigen were determined. In parallel, the binding curve for a mixture of the two populations of antibodies binding to the whole $\beta^S(1\text{-}55)$ was determined experimentally. Then the

two curves for the individual subpopulations of antibodies were combined mathematically according to the above equations. The resulting predicted curve for the mixture of antibodies was found to agree well with the experimental curve for the mixture. Also, the slope for the mixture was found to be increased as predicted. Thus, the theory was shown to be applicable at least in this experimental system.

Significance to Biomedical Research: The theory developed should be useful in understanding the shape of radioimmunoassay binding curves for the many antigens which have multiple determinants.

A major implication of the results is that in many cases for complex antigens one cannot determine meaningful physical parameters such as affinity or association constants from simple graphical analyses such as Scatchard plots. When one needs quantitative binding parameters such as these, it is best to fractionate the antisera to obtain monospecific antibodies to which the mass action law equations can be more readily applied.

Proposed Course of Project: Concluded.

Publications:

Berzofsky, J.A., Curd, J.G. and Schechter, A.N: Probability Analysis of the Interaction of Antibodies with Multideterminant Antigens in Radioimmunoassay: Application to the Amino Terminus of the β Chain of Hemoglobin S. Biochemistry 15 (May, 1976), in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,016-03 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Immunological and Structural Studies of Human Fetal Hb

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

PI:	A. Eastlake	Chemist	LCB NIAMDD
	K. Thomas	Guest Worker	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.2	0.6

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

Human fetal hemoglobin (HbF) has a weaker affinity for 2,3 diphosphoglyceric acid than HbA and other characteristics, such as non-participation in gel formation with HbS, that distinguish it from HbA. The molecular basis of these differences is not clear. HbF contains two α chains and two γ chains instead of the two α chains and two β chains of HbA. As a result there are 39 amino acid differences between HbF and HbA, several of which are in the 2,3 diphosphoglyceric acid binding site. A number of others are clustered in one exposed helical region of the γ chain. We are using solid phase synthetic methods to reproduce this helical region. Immunoabsorption of an antiserum to HbF will then be used in an attempt to obtain non-precipitating antibodies specific for this region which do not crossreact with HbA. A radioimmunoassay using [^{14}C]-carbamoylated HbF will be used to assess the specificity of binding. We have also obtained crystals of HbF of suitable size for X-ray diffraction studies. The specific antibodies and X-ray studies will give information on the conformational and surface structural characteristics of HbF. In addition the antibodies may be useful in quantitating small amounts of HbF in standard laboratory procedures and in prenatal diagnosis of hemoglobinopathies.

PHS-6040
(12-75)

Project Description:

Objectives: Immunologic studies of staphylococcal nuclease and, subsequently, human hemoglobin in our laboratory have used several new approaches. First, synthetic peptides have been produced by a solid-phase method rapidly and in good yield and purified to a high degree. Second, these peptides, representing a portion of the amino acid sequence of the protein, have been insolubilized by attachment to an inert support and used to select from an antiserum to the protein those antibodies which specifically bind without precipitation, in the region of the protein represented by the peptide sequence. Third, a radioimmunoassay has been used to demonstrate the specificity of the binding of such a restricted antibody population by competition between the peptide and the intact protein, and by demonstration that protein molecules with structural alterations (i.e. an amino acid substitution) in the region of antibody specificity do not bind the antibodies.

Human fetal hemoglobin has 39 amino acid differences from HbA some of which are clustered in limited areas of the surface of the molecule. Other substitutions are in the binding site for 2,3-diphosphoglyceric acid. Both the surface interactions and the 2,3-DPG binding affinity of HbF are different from those of HbA. The goal of this project is the isolation of antibodies specific for HbF and their use as probes of the conformation and surface structural features of this molecule. To this end a study of the crystal habit of HbF is also being undertaken.

Methods: The isolation of human HbF from umbilical cord blood samples and sickle cell anemia patients with high levels of HbF has been carried out by standard ion-exchange chromatographic procedures. Peptides have been prepared using a rapid modification of the Merrifield solid-phase method. Gel filtration will be used to purify the peptides. They will be analyzed for completeness of each coupling step by the 2,hydroxynaphthaldehyde method and for overall purity by fingerprinting and amino acid analysis.

Peptides will be coupled to Sepharose beads by CNBr activation. Antisera to HbF will be raised in goats and fractionated on the immunoabsorbents. A radioimmunoassay using ^{14}C-carbamoylated HbF will be developed to assess the specificity of binding of the fractionated antibodies.

Crystallization trials have been carried out using a vapor diffusion method and the conditions described by Perutz for the crystallization of human HbA. Crystallization of oxy-HbF and carbonmonoxy-HbF will be attempted in order to produce a crystal of suitable durability for X-ray study.

Findings: Highly purified HbF has been isolated from cord blood samples. Peptides representing specific regions of the molecule have been synthesized. Crystals of oxy-HbF have been obtained at 4°, 2.5 M phosphate buffer and 4% HbF concentration. Typical crystal sizes are 1x0.6x0.6 mm. These crystals were found to diffract at 4° in preliminary diffraction studies.

Significance to Biomedical Research: The α chain of HbF may have arisen by gene duplication from the β chain of HbA. No differences which might account for the selective advantage of HbF over HbA have been found. Studies

of the conformation and surface structural characteristics of HbF may shed light on these differences. This can be approached by using specific antibodies to HbF and through crystal structure studies. In addition, antibodies specific to HbF may be used to quantitate small amounts of HbF replacing methods now in use which are not highly quantitative. These antibodies may be useful in prenatal of abnormalities of hemoglobin production and sickle cell disease, and in studies of the differentiation of erythroid cells.

Proposed Course: Antibodies to HbF will be raised in goats. Peptides representing specific sequences exposed on the surface of HbF in regions where there are differences from HbA will be used as immunoabsorbants to isolate antibodies specific to HbF which do not cross-react with HbA. HbF will be labelled with $K^{14}CNO$ and used to determine the specificity of the antibodies in a radioimmunoassay.

The collection and analysis of diffraction data on the crystals of HbF will continue. The space group will be determined on a precession camera. If these studies are successful we may pursue an X-ray analysis to high resolution.

Publications:

Young, N.S., Curd, J.G., Eastlake, A., Furie, B. and Schechter, A.N.: Isolation of Antibodies Specific to Sickle Hemoglobin by Affinity Chromatography Using a Synthetic Peptide. Proc. Nat. Acad. Sci. 72: 4759-4763, 1975.

Eastlake, A., Curd, J.G. and Schechter, A.N.: The Amino Terminal Region of the β Chain of Sickle Hemoglobin: I. Synthesis and Purification of Oligopeptides. J. Biol. Chem., in press.

Young, N.S., Eastlake, A. and Schechter, A.N.: The Amino Terminal Region of the β Chain of Sickle Hemoglobin: II. Characterization of Monospecific Antibodies. J. Biol. Chem., in press.

Schechter, A.N., Young, N.S., Curd, J.G., Furie, B. and Eastlake, A.: Synthetic β^S Chain Oligopeptides. I. Use in Affinity Chromatography to Purify Antibodies Specific for Hemoglobin S. in Symposium on Molecular and Cellular Aspects of Sickle Cell Disease (Hercules, J.I., Cottam, G.L., Waterman, M., Eaton, W.A. and Schechter, A.N., Eds.), U.S. Government Printing Office, DHEW Publication No. (NIH) 76-1007, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,017-04 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Immunochemical Studies of tRNA

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

PI:	D. Eilat	Visiting Research Fellow	LCB NIAMDD
	P. DiNatale	Visiting Research Fellow	LCB NIAMDD
	A.N. Schechter	Chief, Sec. on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)
Arthritis and Rheumatism Branch, NIAMDD (Dr. Alfred Steinberg)
Chemistry Branch, NCI (Dr. Dolph Hatfield)
Laboratory of Microbial Immunity, NIAID (Dr. Richard Asofsky)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0

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

We have studied tRNA binding immunoglobulins in the sera of NZB/NZW mice and in patients with systemic lupus erythematosus (SLE) as part of a project to obtain antibodies with conformational specificity for nucleic acids. The mice develop immunoglobulins which bind ^{3}H-tRNA, when they develop a disease process with similarities to SLE and we have been able to isolate these antibodies by affinity chromatography. We have also studied ^{3}H-tRNA binding immunoglobulins by these methods in the sera of several patients with SLE. This binding is better inhibited by single stranded viral RNA than by the tRNA itself.

We examined changes which occur within a group of isoacceptor E. coli tRNAs before and after bacteriophage MS2 infection. The pattern of reversed phase column chromatography is different from that of virus infected E. coli leucyl-tRNA isoacceptors. The RPC-5 pattern of the latter tRNA shows several new peaks of leucyl-tRNA. Aminoacylation and codon recognition studies suggest that these tRNAs are some modified forms of normal leucine tRNA isoacceptors. This modification may be involved in the mechanism of inhibition of host protein synthesis by the virus.

Project Description:

Objectives:

1) To date, over sixty different tRNA molecules have been sequenced and the first crystal structures have been elucidated. Conformational changes of tRNA in solution, during the various stages of protein biosynthesis remains however to be investigated. One approach to this problem is to raise antibodies to tRNA which will be conformationally specific. These antibodies might distinguish between conformations of uncharged tRNA, aminoacyl-tRNA, peptidyl-tRNA, ribosome bound tRNA, etc. The finding of such antibodies could also contribute very much to the general field of protein-nucleic acid interaction, since the stability of such a complex would be expected to be higher than that of many tRNA-enzyme complexes. We have been able to find such antibodies in the sera of NZB/NZW mice and in patients with systemic lupus erythematosus.

2) Regulation of tRNA biosynthesis has been shown in procaryotic and eucaryotic cells following bacteriophage infection and oncogenic virus infection respectively. The MS2 virus-E. coli system provides a unique opportunity to observe this phenomenon, because the sequences of different viral proteins as well as many viral RNA sequences have lately been established. We undertook to look at the specific changes which occur within a few groups of isoacceptor tRNAs, before and after virus infection, and try to relate these changes to the mechanism of inhibition of host protein synthesis by the virus.

Methods Employed: Radioactive ^{3}H-tRNA was prepared by isolating tRNA from Q 13 E. coli cells grown on ^{3}H-uridine containing medium. The radioimmunoassay used for detection of antibodies included Sephadex chromatography of ^{3}H-tRNA·antibody complexes. Ribonuclease activity was removed from serum by charcoal treatment or by gel filtration. tRNA-Sepharose affinity columns were prepared by direct linking of tRNA to CNBr activated Sepharose or by reacting periodate-oxidized tRNA with Sepharose dihydrazide, followed by borohydride reduction. Identification of immunoglobulin class of the tRNA antibody was performed by immunoelectrophoresis using specific goat anti-mouse immunoglobulins and ^{125}I-tRNA. Fab and Fc fragments from mouse IgG were prepared by papain digestion and were separated by preparative electrophoresis on agar. E. coli C-3000 were grown and infected with MS2 virus in the NIH fermenter unit. The tRNA was extracted from preinfected and postinfected cells, and the patterns of isoacceptors were determined by RPC-5 reversed phase chromatography. Coding properties of tRNA isoacceptors were determined by using trinucleotides or polynucleotides and ribosomes in the nitrocellulose filter assay.

Major Findings: A high affinity binding activity to native tRNA was found in old NZB/NZW mice which have an autoimmune disease, very similar to systemic lupus erythematosus (SLE) in humans. The appearance of the tRNA antibody at about 4 1/2 months of age in female mice correlates well with the development of the disease. Maximum amounts of tRNA antibody are found between 7 and 10 months of age. The antibody can bind all added tRNA and is not specific for a single class or a specific tRNA.

Sephadex G-200 column chromatography, elimination of immunoglobulins by class specific anti-mouse immunoglobulins and immunoelectrophoresis have shown that most of the binding activity is IgG immunoglobulin. Fab and Fc fragments were prepared from NZB/NZW IgG immunoglobulins and are currently being tested for binding activity. The murine anti-tRNA antibody was purified about 750-fold by affinity chromatography using Sepharose column to which periodate oxidized tRNA was attached. The bound antibody was eluted with 4 M guanidine hydrodhloride. A different affinity column prepared by direct reaction of tRNA with CNBr activated Sepharose gave only 6-fold purification.

We have also found a similar tRNA binding activity in some patients with SLE. Here too, the activity was associated with the IgG immunoglobulins. The specificity of the human antibody seems to be somewhat different from that of the mouse antibody. Competition experiments showed that nucleotides, synthetic polynucleotides and DNA were not effective inhibitors, but viral RNA inhibited the tRNA·human antibody complex formation better than the tRNA itself. The human antibody was also found to be more specific for the native conformation of the viral RNA than for the native conformation of tRNA.

E. coli tRNA specific for leucine was investigated before and after MS2 bacteriophage infection. The isoacceptor tRNA distribution on a reversed phase column has been drastically changed. Three new peaks of leucyl tRNA could be detected following infection. The coding specificities of the new isoacceptors were studied using trinucleotides, polynucleotides and ribosomes. From these and aminoacylation studies it appears that the new tRNAs are some modified forms of normal leucine tRNA isoacceptors. One of the leucine tRNA isoacceptors, $tRNA_3^{Leu}$ was found to bind very tightly to the virus particles themselves. These two phenomena may be involved in the mechanism of inhibition of host protein synthesis by the virus.

Significance to Biomedical Research: The tRNA-antibody project may lead to new information on the origin, development, and genetics of certain autoimmune diseases, particularly systemic lupus erythematosus. The tRNA regulation project will provide a better understanding of the cellular mechanisms following viral infection.

The development of antibodies to tRNA, and other nucleic acids, offers great potential for studying the conformation of nucleic acids (by methods analogous to those we have used for studying the conformation of proteins) and for probing various questions related to the structure and function of nucleic acids in the ribosome, nucleus and in several biological processes. The characterization of these NZB/NZW mice antibody proteins is a start in this direction.

Proposed Course of Project:

1) Further characterization of the tRNA antibody with respect to the specific recognition site which will be attempted. The human antibodies will be purified by affinity chromatography and compared to the purified mouse antibody. Myeloma proteins from NZB mice will be screened for tRNA binding activity. A search for new assays for nucleic acid antibodies from different sources will be carried out. The structure of tRNA during various stages of protein synthesis will be investigated.

2) The leucine tRNA isoacceptors from post infection E. coli cells will be purified and subjected to structural analysis in order to establish their role in the infection process of bacterial cells by MS2 virus.

Publications:

Di Natale, P., Schechter, A.N., Lepore, G.C., and De Lorenzo, F.: Histidyl Transfer Ribonucleic Acid Synthetase from Salmonella typhimurium: Interaction with Substrates and ATP Analogues. Eur. J. Biochem. 62: 293-298, 1976.

Eilat, D., Schechter, A.N. and Steinberg, A.D.: Antibodies to Native tRNA in NZB/NZW Mice. Nature 259: 141-143, 1976.

Di Natale, P. and Eilat, D.: Patterns of E. coli Leucine tRNA Isoacceptors Following Bacteriophage MS2 Infection. Nucleic Acids Research 3: 917, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,018-01 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Does Freund's Adjuvant Denature Protein Antigens? An EPR Study of Emulsified Hemoglobin

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

PI:	J.A. Berzofsky	Research Associate	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)
Laboratory of Chemical Physiology, NIAMDD (Dr. Hideo Kon)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

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

Use of complete Freund's adjuvant for production of antibodies to study protein conformation is valid only if emulsification in adjuvant does not denature protein antigens. Using electron paramagnetic resonance to observe directly the protein in situ in the opaque emulsions, we demonstrate that hemoglobin is not denatured by emulsification or storage in adjuvant for 24 hours at 4°C. The results also rule out one proposed mechanism of adjuvant action, via alteration of protein structure, at least in this case.

PHS-6040
(12-75)

Project Description:

Objectives: To use antibodies made to proteins in complete Freund's adjuvant (CFA) as probes of native conformation, it is important to know that emulsification in adjuvant does not denature the protein. Either surface denaturation in formation of the emulsion, or denaturation by components of the adjuvant (mineral oil, an emulsifying agent, and killed tubercle bacilli) could occur. Optical probes usually used to monitor protein conformation, such as circular dichroism, optical rotatory dispersion, fluorescence, and optical absorption, are impossible to use in an opaque emulsion. However, hemoglobin, a moderately labile protein, can be studied by electron paramagnetic resonance (EPR) without hindrance by the emulsion. We investigated the state of hemoglobin after emulsification in CFA.

Major Results and Methods: Oxy and high spin ferric hemoglobin were each emulsified in CFA and stored for 2 hrs or 24 hrs at 4°C before freezing in liquid N_2 for observation by EPR at liquid helium temperatures (about 8-9° K used here). These were compared with identical samples not emulsified in adjuvant. Neither the oxy nor the ferric (met-) hemoglobin showed any detectable denaturation after either time period. Controls as well as previous published work demonstrated that even a small extent of denaturation perturbing the heme cavity would have been easily detected. Furthermore, the oxyhemoglobin did not even undergo any oxidation to ferric (met) hemoglobin - one of the first signs of disturbance of the protein conformation. Thus we conclude that emulsification in CFA does not denature hemoglobin at least up to 24 hrs at 4°C.

Significance to Biomedical Research:

1) Those who use antibodies made to proteins emulsified in complete Freund's adjuvant as probes of protein conformation may now have more confidence that the form of the protein used as immunogen is still native.

2) The sometimes proposed mechanism of adjuvant action involving change in protein conformation has been shown not to apply, at least in this case.

Proposed Course of Project: Concluded.

Publication:

Berzofsky, J.A., Schechter, A.N. and Kon, H.: Does Freund's Adjuvant Denature Protein Antigens? EPR Studies of Emulsified Hemoglobin. J. Immunol. 116, 270-272, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,019-01 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Intercalation Chromatography

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

PI:	K. Thomas	Guest Worker	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	0

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

The ethidium cation of the salt ethidium bromide has been previously shown by others to be a sequence specific nucleic acid intercalating agent. We have covalently linked the ethidium cation to agarose gel beads through a spacer arm to produce an affinity chromatography system for nucleic acid. The interaction of the gel bound ethidium ion into nucleic acid double helices has been demonstrated by a variety of biophysical techniques including fluorescence. Preliminary experiments indicate that crude transfer ribonucleic acid can be fractionated to some degree on this column using elution with a linear sodium chloride gradient.

PHS-6040
(12-75)

Project Description:

Objectives: Many planar polycyclic aromatic compounds, including those of the phenanthrilic class such as the ethidium cation, have been demonstrated to intercalate (i.e. insert) between adjacent base pairs in nucleic acid double helices. Recent solution results indicate that the ethidium cation can discriminate various intercalation sites that are formed by different combinations of base pairs in such helices. This sequence specific recognition of double helical polynucleotides is thus a potentially useful physical basis for the fractionation of these molecules. Therefore, we have begun an investigation of the potential use of the ethidium cation covalently linked to agarose to fractionate nucleic acids.

Methods Employed: Cyanogen bromide activated agarose was reacted with 3,3'-diaminodipropylamine followed by succinic anhydride to produce a "spacer arm" with a terminal carboxylic acid group. The ethidium cation was covalently linked to this carboxyl group by utilizing a water soluble carbodiimide mediated coupling. A column that was packed with this material has been used to bind a crude mixture of E. coli transfer ribonucleic acid (tRNA) species. The ethidium column was eluted with a linear sodium chloride gradient at room temperature. The fractions were assayed by a radioactive tRNA aminoacyl charging technique. The complex of tRNA with the ethidium gel was also measured by both direct spectrophotometric and fluorescence measurements of the gel. Equilibrium partition measurements were made on a tRNA solution in the presence of the ethidium gel.

Major Findings: E. coli methionine and phenylalanine tRNAs are clearly separated from each other by sodium chloride linear gradient elution. The sodium chloride sensitivity of the ethidium-nucleic acid complexes in the early eluting methionine tRNA and the latter eluting phenylalanine trna fractions were compared in solution after removal of the elution salt by dialysis. The ethidium complex of the earlier eluting fraction was dissociated by a lower concentration of sodium chloride than the latter eluting fraction which is thus consistent with the behavior of these fractions on the column. The fractionation was demonstrated to be a result of the bound ethidium interacting with the nucleic acid since a control column, identical to the ethidium column except that the ethidium group at the end of the 'spacer arm' was replaced by an ethylamine group, did not retard the tRNAs of salt concentrations well below those necessary to elute the ethidium column. The basis for fractionation was shown to involve intercalation of the covalently bound phenanthriline ring into the nucleic acid double helical structure since the ethidium fluorescence was enhanced upon binding tRNA, a physical parameter previously determined to be indicative of the intercalation complex. Furthermore, fluorescence enhancement, absorbance maximum red shift, equilibrium partition binding and the dynamic flow binding of the tRNA to the gel paralleled each other in titration of complex formation with tRNA and subsequent complex dissociation by sodium chloride.

Significance to Biomedical Research: The principle of intercalation chromatography may be useful for the fractionation of nucleic acids. Further-

more, since many intercalating compounds, including salts of the ethidium cation, are used as drugs and are thought to mediate their effect through interaction with nucleic acids by intercalation the elucidation of the specificity of this interaction is necessary to the understanding of the detailed mechanism of action of these compounds. Also, since it has been proposed that surface aromatic amino acids on proteins that interact with nucleic acids may participate in intercalation complexes with the nucleic acid double helices, the investigation of the discriminatory potentials of the intercalation process could have relevance to the more general area of specific protein-nucleic acid recognition which is of crucial importance for normal cell function in the defense of cells against foreign genetic material.

Proposed Course of Project:

1) Other tRNAs will be located in the elution profile and various conditions will be tested in an attempt to characterize elution parameters that will optimize the fractionation potential of the column.

2) We will investigate the interaction of other nucleic acids such as double and single stranded homogeneous digonucleotides and DNA molecules in the process of further characterizing the specificity of the intercalation process on the column.

3) Specific elution with ethidium bromide itself and potential competitive molecules including aromatic amino acids will be attempted to help clarify the generality of this type of interaction.

4) We intend to test the ability of this column to separate nucleic acids from crude cell extracts.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,020-01 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Radioimmunoassay of Creatine Kinase: A Model for Radioimmunoassay of Human Serum Enzymes

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

PI: Alan N. Schechter Chief, Sec. on Macromolecular Biology LCB:NIAMDD

COOPERATING UNITS (if any)
Clinical Pathology Dept., Clinical Center (Dr. Andre c. van Steirteghem and Dr. Mark H. Zweig)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD:NIH; Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

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

Human serum enzyme assays for clinical diagnosis usually make use of measurements of catalytic activity. In many situations, this may not be ideal; e.g. isoenzymes reflecting different damaged tissues may be indistinguishable or inactive enzymes may be released. Radioimmunassay offers a very sensitive and specific way in which to measure enzyme concentration and to distinguish isoenzymes. Creatine kinase was chosen as a model for this approach because of its clinical importance and its simple isoenzyme distribution. The enzymes from human skeletal muscle and brain have been purified, used as immunogens to raise antibodies, and labeled with ^{125}I. Radioimmunoassays for each of these enzymes have been developed and are now being applied in clinical diagnostic studies.

PHS-6040
(12-75)

Project Description:

Objectives:

There are sound theoretical reasons for believing that direct measurement of enzyme molecule concentrations in human serum may have significant advantages over measurements of enzyme activity for diagnostic studies. Enzyme assays are insensitive to fragments of molecules or other inactive species and do not distinguish among isoenzymes that may come from different tissues. Radioimmunoassay has the potential of great sensitivity and specificity, including, frequently, the ability to discriminate among isoenzymes. Further, the immunological characteristics of a protein may be detectable in incomplete or inactive molecules.

In order to begin a study of the clinical potential of radioimmunoassay of human serum enzymes, we have purified creatine kinase and developed a radioimmunoassay. This radioimmunoassay is now being applied in several clinical applications.

Methods Employed:

Human brain and skeletal muscle were homogenized, extracted, and the creatine kinase isoenzymes were purified by ethanol and ammonium sulfate precipitation, anion exchange chromatography, and gel filtration. The purified enzymes were characterized by disc gel electrophoresis and amino acid analysis.

Sheep and rabbits were immunized by repeated injection of these purified enzymes in Freund's adjuvant. Antibody production was followed by Ouchterlony quantitative precipitation and immunoinhibition assays.

The enzymes were labeled with ^{125}I by the Bolton-Hunter acylation reagent and purified by gel filtration. A double antibody radioimmunoassay was developed for the primary antibodies with burro-anti-sheep and sheep-anti-rabbit gamma globulin.

Major Findings:

The enzyme purification steps yielded about 10 mg of essentially pure muscle creatine kinase and about 40 mg of essentially pure brain creatine kinase. The enzymes behaved as single bands on disc gel electrophoresis and the amino acid composition was similar to that previously reported for these enzymes. The molecular mass of each was about 80,000 daltons as determined by SDS-poly-acrylamide electrophoresis.

Antibodies to each were obtained in good titer. ^{125}I labeling of each enzyme was accomplished with the Bolton-Hunter reagent and a double-antibody radioimmunoassay was developed. This radioimmunoassay is now being standardized for use in clinical assay of human serum samples and in other diagnostic applications, such as in assay of tissue culture enzymes.

Significance to Biomedical Research:

Measurement of creatine kinase serum enzyme molecules rather than catalytic activity should provide more useful clinical information about tissue damage in a variety of contexts. The radioimmunoassay's sensitivity and ability to distinguish among creatine kinase isoenzymes should offer new diagnostic potentials. These methods should be applicable to other human enzymes now routinely measured.

Proposed Course of Project:

These radioimmunoassays will be used in parallel to standard enzyme assays in the Clinical Pathology Department to assess the utility of this new method. The radioimmunoassay will be applied in other clinical situations, such as tissue culture means of detecting muscular dystrophy heterozygotes. Development of radioimmunoassays for other clinically significant isoenzyme systems, such as aspartate amino transferase, will be initiated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,021-01 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies of the Kinetics of Gelation of Sickle Cell Hemoglobin: Peptides as Possible Inhibitors

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

PI:	C.T. Noguchi	Guest Worker	LCB NIAMDD
	A. Eastlake	Chemist	LCB NIAMDD
	A.N. Schechter	Chief, Sec. on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	

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

The gelation kinetics and solubility assay for deoxygenated hemoglobin S developed by Hofrichter, Ross and Eaton is being used to study the effect of amino acids, dipeptides and oligopeptides on gelation. The delay time of gelation is determined by monitoring turbidity changes at 800 nm. The solubility is determined from the supernatant infra-red spectra of the sample after the gel is sedimented at high speed in the ultracentrifuge. The anti-sickling effect of amino acids, dipeptides and oligopeptides can be determined by the amount of retardation of gelation and the increase of solubility of deoxygenated hemoglobin S. The ultimate goal of this project is to study the surface interactions of deoxygenated hemoglobin S and to find a therapeutic anti-sickling agent.

PHS-6040
(12-75)

Project Description:

Objectives: The study of the gelation process of sickle cell hemoglobin has been of continuting interest to this laboratory. We have focused on the kinetics of the gel formation and solubility of sickle cell hemoglobin. Studies on nuclease and other proteins demonstrating the complementing effect of protein fragments have suggested to us that hemoglobin fragments may indeed affect the gelation of sickle cell hemoglobin. If the mechanism of gel formation can be sufficiently retarded, it is believed that the tendency of a sickle-red blood cell to deform or sickle can be reduced or eliminated. The goal of this project is to study the effect of amino acids, dipeptides and oligopeptides (chosen from the normal and sickle hemoglobin sequence) on the kinetics and solubility of sickle hemoglobin.

Methods Employed: Large amounts of hemoglobin S have been prepared by ion exchange chromatography from units of SS blood. The method of optically monitoring turbidity and ultracentrifugation developed by Hofrichter, Ross and Eaton has been adopted. In this assay, concentrated hemoglobin S (similar to the concentration found in red blood cells) is deoxygenated with sodium dithionite in a tube sealed under nitrogen. The kinetics of gelation are monitored by observing turbidity changes at 800 nm at a controlled temperature - a delay period in gelation is usually observed and measured. The sample is then spun in a thermally controlled ultracentrifuge at high speed which collapses the gel into a pellet leaving a solution saturated with tetrameric hemoglobin. The hemoglobin saturation at that temperature is obtained from measurements on a Cary 17 of the supernatant concentration.

Fragments of the amino terminal end of beta S hemoglobin and several other sites of beta hemoglobin believed to participate in gel formation have been synthesized by the Corley-Sachs modification of the Merrifield method.

At these high concentrations of hemoglobin additives may have complex effects due to non-ideality. Thus we have begun these studies with simple amino acids to analyze these effects.

Major Results: Various amino acids have been tested in the gelation assay of Hofrichter, Ross and Eaton. Several amino acids had no apparent effect on gelation or the saturation of hemoglobin S. A few exhibited the ability to slightly retard gelation and increase hemoglobin S solubility. Phenylalanine was the only amino acid of many tested that significantly affected gelation and solubility.

The interpretation of these data will depend on the results of further studies investigating other amino acids. Current studies in progress also include investigating the dependence of gelation and solubility of hemoglobin S on the concentration of amino acids (phenylalanine and tyrosine) and on the length of a homopolymer (polylysine, for example).

When these basic data on the effects of amino acid and peptide additives are complete, we will begin a systematic study of the peptides from the contact regions which have been synthesized as possible stereospecific inhibitors of gelation. If indicated, studies of erythrocyte permeability and physiology (oxygen affinity, pH, etc.) will be done with selected peptides.

Significance to Biomedical Research: Peptide-protein interactions can be used as a probe to study the surface structure of normal and mutant hemoglobins. Of particular interest is hemoglobin S which aggregates to form a viscous gel under deoxygenated conditions. Increasing the delay time of gelation and solubility of hemoglobin S are the minimal criteria for a suitable drug in sickle cell anemia therapy. So far, we have found that some amino acids have the anti-sickling tendency. By taking peptides from the hemoglobin sequence, we hope to increase the specificity for anti-sickling agents, we hope to obtain information about hemoglobin-hemoglobin interactions, which might prove useful in studying other hemoglobinopathies besides sickle-cell anemia.

Proposed Course of Project: The gelation and solubility studies on hemoglobin S will continue and the synthesized oligopeptides will be tested. The initial data on the amino acids will be used as background data to which oligopeptide results will be compared. The ultimate goal of the project will be to find an antisickling agent with high specificity which can effectively penetrate the erythrocyte but with minimal side effects, such as change in hemoglobin oxygen affinity.

Publication:

Schechter, A.N.: Synthetic β^S Chain Oligopeptides. II. Use as Potential Stereospecific Inhibitors of Hemoglobin S gelation. In Hercules, J.I., Cotton, G.L., Waterman, M.R., Eaton, W.A. and Schechter, A.N. (Eds.): Symposium on Molecular and Cellular Aspects of Sickle Cell Disease. U.S. Govt. Printing Office, DHEW Publication No. (NIH) 76-1007, in press.

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

I. PROTEIN STRUCTURE AND MECHANISM OF ENZYME AND HORMONE ACTION

Aminoacyl tRNA Synthetases

Over a period of years we have accumulated evidence that the glycyl tRNA synthetase of yeast can exist in two forms. These are present in the cells and vary in concentration in a reciprocal manner during growth. During the past year we have sought to study the cyclic interconversion of the two forms in a cell-free extract, and this project is approaching a stage where fruitful experimentation will be possible.

. . . . . Dr. S. Black and Mrs. B. Hazel

Studies Concerning the Mechanism of Exophthalmos and of Thyrotropin Receptor Function

In studies reported this year we have shown the following:

(a) The tryptic fragment of the TSH receptor has been purified. It has a molecular weight of 24,000, contains 30% carbohydrate, and has a 10% sialic acid content. The binding activity of the receptor fragment is lost when it is exposed to beads of neuraminidase-Sepharose or concanavalin A-Sepharose.

(b) Studies on human retro-orbital tissue adipocytes were performed. ^{125}I-TSH binding to these adipocytes was significantly enhanced by the autoimmune gamma globulin as compared to normal gamma globulin at the same concentration. In contrast, control experiments using dog retro-orbital tissue adipocytes showed that ^{125}I-TSH could be bound but that there was no significant increase by the different gamma globulin preparations. Data using human adipocyte membranes were analogous.

(c) Supernatant solutions from crude human and guinea pig membrane preparations have been shown to contain a thermolabile, nondialyzable inhibitor of thyrotropin binding which acts by forming a thyrotropin-inhibitor adduct rather than by directly interacting with the membrane receptors, i.e., a soluble cytoplasmic TSH binding factor has been described.

(d) Gangliosides have been shown to inhibit ^{125}I-labeled thyrotropin binding to thyrotropin receptors. The inhibition is hormonally specific and results from an interaction between the ganglioside and TSH. The inhibition is associated with a specific alteration in the conformation of the TSH molecule.

(e) The B chain of cholera toxin and the β subunits of thyrotropin, luteinizing hormone, human chorionic gonadotropin, and follicle-stimulating hormone have been shown to have a region of sequence analogy believed to correlate with their ability to bind to receptors on cell membranes.

(f) Thyrotropin and cholera toxin have been shown to have an analogous mode of interaction with receptors on thyroid plasma membranes, and a mechanism by which TSH transmits its message to the cell machinery has been detailed.

(g) Plasma membranes derived from a rat thyroid tumor which is unresponsive to thyrotropin but not to dibutyryl cyclic AMP have been shown to have no gangliosides capable of interacting with TSH by comparison to normal rat thyroid.

(h) Patients with Graves' disease and exophthalmos demonstrate delayed hypersensitivity to antigens present in extracts of certain normal human tissue, namely, thyroid gland and retro-orbital tissue. The antigen has been purified and characterized as thyroglobulin.

. . . . . Drs. L. D. Kohn, S. M. Aloj, P. H. Fishman (Developmental and Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke), F. D. Ledley, G. Lee, M. F. Meldolesi (Clinical Endocrinology Branch, NIAMDD), B. R. Mullin, and R. J. Winand (University of Liege, Liege, Belgium)

Studies on the Mechanism of Action of Interferon

Cholera toxin and TSH inhibit the ability of interferon to establish its antiviral activity in mouse L-cells. The presumption of these experiments was that by interacting with gangliosides or ganglioside-like oligosaccharides on the L-cell membrane, the cholera toxin and TSH might either directly or indirectly interfere with ganglioside or ganglioside-like oligosaccharide structures believed important to interferon interactions with its cell membrane receptors. Direct *in vitro* binding experiments already indicate that this presumption is valid.

. . . . . Drs. L. D. Kohn and R. M. Friedman (Laboratory of Experimental Pathology, NIAMDD)

Studies Concerning the Biosynthesis of Collagen

A procollagen peptidase activity for the C terminal extension peptide of type I procollagen has been defined.

. . . . . Drs. L. D. Kohn and G. Lee

Studies Concerning Membrane Structure and Function

An improved purification procedure (using hydrophobic chromatography and antibody-Sepharose columns) has been worked out for D-lactic dehydrogenase.

. . . . . Drs. L. D. Kohn and H. R. Kaback (The Roche Institute of Molecular Biology, Nutley, New Jersey)

Tryptophan Synthase of Escherichia coli

Chemical and physical methods are being used to compare the isolated α and $\beta 2$ subunits of tryptophan synthase with the reconstituted $\alpha_2\beta_2$ complex.

Important findings are that essential histidyl and sulfhydryl groups in the active site of the β_2 subunit can be modified in the free native β_2 subunit but not in the native $\alpha_2\beta_2$ complex and that the cofactor, pyridoxal 5'-phosphate is bound reversibly to the β_2 subunit but irreversibly to the $\alpha_2\beta_2$ complex. These results indicate that the active site of the β_2 subunit becomes partially shielded from the aqueous environment in the $\alpha_2\beta_2$ complex and may be located in a common subunit interaction site.

Other studies show that low concentrations of salts promote subunit association, presumably by neutralizing the mutually repulsive ionized groups in the interaction sites of the α and β_2 subunits. Effects of salts on the binding of pyridoxal 5'-phosphate are also being studied.

Studies with substrate analogs are aimed at further elucidating the mechanism and active site groups of tryptophan synthetase. *Trans*-L-2-amino-4-methyl-3-butenoic acid irreversibly inhibits the $\alpha_2\beta_2$ complex and causes striking spectral changes. A new type of pyridoxal-P dependent reaction has been discovered to be catalyzed by tryptophan synthetase: the conversion of two β,γ-unsaturated amino acids to corresponding saturated α-keto acids. This discovery further extends our understanding of the mechanism of pyridoxal-P catalyzed reactions.

. . . . . Dr. E. W. Miles

II. SYNTHESIS OF PROTEINS AND NUCLEIC ACID

Nucleic Acid Synthesis

Replication of linear duplex T4 DNA *in vivo* requires several proteins encoded by the phage DNA including T4 DNA polymerase, the T4 DNA unwinding protein (gene 32 protein), and the proteins encoded by genes 44, 62, 45, and 41. The complex of the gene 44 and 62 proteins is a DNA-dependent ATPase (or dATPase) whose activity is enhanced by the gene 45 protein. During the past year we have developed a reliable complementation assay for 41 protein and have shown that the protein purified using this assay has a nucleotidase activity hydrolyzing both ribo- and deoxyribotriphosphates which requires single-stranded DNA. With low concentrations of T4 DNA polymerase, the copying of single-stranded linear or circular templates *in vitro* is strongly dependent on the addition of the 32, 44/62, 45, and 41 proteins. The same reactions can be catalyzed by high concentrations of the T4 DNA polymerase alone. With circular DNA's, the product strand is not covalently attached to the template. A large part of the product is nicked duplex circles which can be sealed by *Escherichia coli* or T4 DNA ligase. The mechanism of chain initiation in this system is not yet clear. Nicked linear duplex DNA molecules are not copied by the T4 polymerase alone, but do serve as a template in the presence of the gene 32, 44/62, and 45 proteins.

We have carried out a large scale search for temperature-sensitive mutants of *E. coli* which enhance the frequency of replication errors (mutator mutants), using a new method suitable for screening large numbers of clones in which lac^+ mutants arising in a lac^- colony are identified as white papillae on the

surface of red colonies growing on tetrazolium plates. Mutator strains are easily identified by this procedure, but only one of these mutators has proved to be temperature sensitive. This mutation (mut 3) maps at or very close to pol C, the locus which codes for DNA polymerase III, the polymerase required for *E. coli* DNA replication. Mut 3 may constitute a new locus, however, since we have so far failed to find a defect in its DNA polymerase III activity *in vitro*. We have also looked for secondary mutations which would enhance the mutator activity of mut 3. We have found one such mutant which by itself is a relatively weak mutator, but which gives very high mutation frequencies when combined with either mut 3 or with mutants in DNA polymerase III.

. . . . . Drs. N. G. Nossal and E. B. Konrad

Protein Synthesis

Work from the laboratory has established that the peptide chain elongation factor (EF) Tu is present at considerable molar excess over ribosomes. This finding which has recently been confirmed by reports from several other laboratories, contrasts with the results found for EF-G and EF-Ts which are present at a 1 : 1 molar ratio to ribosomes, and raised several questions which are currently being investigated: (1) Do all the molecules of EF-Tu have the same structure and function? (2) How does the cell coordinate the synthesis of EF-Tu, EF-G, and EF-Ts and ribosomes but still maintain the molar excess of EF-Tu?

Recent data indicate that EF-Tu exhibits microheterogeneity, suggesting that all the molecules of EF-Tu are not the same. This finding may be related to the recent discovery by Nomura and coworkers that there are two genes for Tu in *E. coli*, and work is now underway to compare the products of these two genes by tryptic-peptide maps.

Pertaining to the second question, we have recently shown that synthesis of the elongation factors are under control of the relA gene. In the course of these studies we have also shown that the synthesis of a great number of nonribosomal proteins are affected by expression of the relA allele. The data suggest that ppGpp inhibits or stimulates the transcription of many bacterial genes. Work on this phenomenon is continuing, and we have most recently shown that high levels of ppGpp stimulates the synthesis of some amino acid biosynthetic enzymes but not of others.

. . . . . Dr. A. V. Furano

Protein Methylation

By use of our recently developed rapid and easy method for the analysis of methylated amino acids in proteins, we showed that in contrast to the rich array of methylated proteins in the various subcellular fractions of eukaryote, only *E. coli* 50 S ribosomal proteins contained methylated amino acids. However, recent evidence by others has shown that a rapidly metabolized methylated protein may be involved in the chemotactic response of bacteria. These findings have prompted new studies on our part to examine the question of reversible methylation of proteins in *E. coli*.

. . . . . Dr. A. V. Furano

III. COMPLEX CARBOHYDRATES

A cold agglutinin (McC) was isolated from serum of a patient with Waldenström's macroglobulinemia by affinity chromatography using temperature elution from a column of glutaraldehyde-fixed erythrocyte stroma mixed with celite. The agglutinin, which is an IgM kappa, reacts weakly with normal adult erythrocytes and strongly with cord cells, as well as with adult O_i cells, and, therefore, by definition has "anti-i" specificity. The reactivity of all cell types tested increased to approximately the same level after treatment of the cells with ficin. The purified cold agglutinin forms precipitin lines with polylysine conjugates containing oligosaccharides with the non-reducing sequence Galβ1-4GlcNAcβ1-3Gal.... Agglutination of erythrocytes at 4° is specifically inhibited by oligosaccharides with the same non-reducing sequence and by "paragloboside," a glycosphingolipid extracted from human erythrocyte membranes with the structure Galβ1-GlcNAcβ1-3Galβ1-4Glc-ceramide.

In contrast, another cold agglutinin (Ma) which agglutinates adult erythrocytes in preference to cord blood erythrocytes and therefore has anti-I specificity, precipitates only with polylysine-oligosaccharide conjugates containing the non-reducing sequence Galβ1-4GlcNAcβ1-6Gal.... This structure occurs in adult erythrocyte membranes in glycosphingolipids containing ten monosaccharide residues. Ficin treatment produces little change in reactivity of adult cells with Ma. It has been observed previously that glycosphingolipids with four or fewer glycosyl residues tend to behave as cryptic receptors in adult cells in that they bind antibodies only after treatment of the cells with proteolytic enzymes, while the same molecules in fetal cells bind antibodies without proteolysis. These results suggest that the chain length of carbohydrates on surfaces of adult and fetal cells might explain the differential expression of receptors for "anti-I" and "anti-i" agglutinins on human erythrocytes.

. . . . . Drs. V. Ginsburg, D. A. Zopf, and C.-M. Tsai

IV. CELL SURFACES

Structure and Assembly of E. coli Membranes

The surface of *E. coli* consists of an inner or cytoplasmic membrane, a rigid cell wall, and an outer membrane. The outer membrane is relatively simple in composition, is assembled from molecules of protein, lipid, and lipopolysaccharide (LPS) made elsewhere in the cell, and therefore serves as a good model for studying membrane structure and assembly.

Previous work from this laboratory indicated that there are about 10-50 insertion points in the outer membrane into which newly synthesized LPS is inserted, and from which it moves elsewhere in the membrane. Current experiments have used density labeling of such membranes to indicate that, after an initial period, in the outer membrane, the LPS becomes immobilized into "domains" that do not mix with subsequently incorporated LPS. The size and nature of these "domains" are being analyzed.

. . . . . Dr. L. Leive

The outer membrane appears by several criteria to have true membrane (lipid-protein bilayer) character. However, several reports have suggested that lipid mobility in this membrane may be much more restricted than in the cytoplasmic membrane of these and other cells. To determine whether the LPS of this membrane affects the mobility, electron spin resonance spectral analysis was performed. Outer membrane preparations containing LPS with a long polysaccharide chain were much less fluid than preparations containing LPS with a short polysaccharide chain, and treatment with EDTA to reduce LPS content resulted in increased mobility. These results indicate that LPS, and especially its polysaccharide moiety, directly or indirectly causes the restricted mobility of the lipid hydrocarbon chains in the outer membrane.

. . . . . Drs. L. Leive and S. Rottem (The Hebrew University, Jerusalem, Israel)

Physiology and Genetics of an E. coli Strain Important in Studies of Lipopolysaccharide and Membrane Assembly

E. coli O111:B4 is of great interest both because it is the most common of the *E. coli* strains that are enteropathogenic for infants and because it has a very well-characterized LPS with a very long polysaccharide chain. For the latter reason it has been used for studies of the outer membrane in several laboratories. Such work has been hindered because standard genetic manipulations have been heretofore impossible in this strain. We have now worked out procedures for conjugation involving production of unstable heterogenotes (stable recombinants cannot be formed) and have derived substrains which can be transduced. We are currently mapping a large number of loci and comparing them to their positions in *E. coli* K12.

. . . . . Drs. L. Leive and W. G. Coleman, Jr.

E. coli O111:B4 has been occasionally reported in the literature to carry a lysogenic phage, or a colicin, or both. We have now characterized a particle carried by our strain of *E. coli* O111:B4. This particle has the morphological characteristics, on electron microscopy, of the well-known transducing phage P1, but differs from it in antigenic specificity. It cannot plate well on any strain yet tested, but shows very high efficiency of generalized transduction into many strains, including *E. coli* K12. It therefore manifests the characteristics of a defective transducing phage. It is of interest because it may transduce a smaller portion of the genome than phage in current use and therefore may enable more precise fine-structure mapping.

. . . . . Drs. L. Leive and W. G. Coleman, Jr.

Phosphatidylserine Synthetase Mutants of E. coli

Mutants of *E. coli* defective in CDP-diglyceride : L-serine phosphatidyltransferase (phosphatidylserine synthetase) can be isolated by a rapid autoradiographic screening assay described previously (Raetz, C. R. H., Proc. Nat. Acad. Sci. U.S.A., 72: 2274-2278, 1975). Four organisms of this kind have now been characterized. The gene (designated pss) which is altered in these

mutants is closely linked to the nadB locus near minute 49 on the *E. coli* chromosome.

Strains carrying the pss-8 mutation do not grow at elevated temperatures and have low levels of an altered synthetase in cell extracts. An analysis of several hundred transductants and temperature-resistant revertants reveals that the pss-8 mutation is responsible both for the enzyme defect and for the phenotype. When a pss-8 mutant is shifted to the nonpermissive temperature, the cells stop dividing and form long filaments. After 3 hours at 44° the level of phosphatidylethanolamine drops from 66% to 32% (percentage of the total lipid phosphorus), while the combined levels of phosphatidylglycerol and cardiolipin rise from 34% to 68%. The antibiotic sensitivity of these mutants is significantly altered.

. . . . . Dr. C. R. H. Raetz

V. MECHANISMS OF INHERITANCE IN *SACCHAROMYCES CEREVISIAE*

The "Killer Character" of Saccharomyces cerevisiae

Strains of the eukaryote *S. cerevisiae* carrying a 1.7×10^6 dalton double-stranded RNA plasmid secrete a toxin which is lethal to strains not carrying this plasmid. Since our last report, we have defined by mutation six new chromosomal genes needed to replicate or maintain the "killer" plasmid, called mak4 through mak9. These have been located on the genetic map: mak4 and mak5 are on chromosome II, mak6 is near mak3 on chromosome XVI, mak7 is on chromosome VIII, mak8 is on chromosome XV near mak1, while mak9 is on chromosome XI. All mak mutants lose the 1.7×10^6 dalton ds RNA but retain 3.0×10^6 and 3.8×10^6 dalton species present in all yeast strains.

We have also detected a new class of dominant chromosomal changes whose effect is to bypass the killer plasmid's need for one or more mak genes for its replication. These new chromosomal changes (called KRB for killer replication bypass) are all tightly centromere linked (two are at the centromere of chromosome X), and are remarkably unstable. They are not translational suppressors. The KRB change might represent either an integrated DNA copy of the killer ds RNA genome or a derepressed alternate plasmid replication apparatus. These and other possibilities are under study.

The m gene is a gene of the mak type normally needed for replication of the killer ds RNA plasmid. We have isolated several strains which are killers in spite of carrying a mutation in the m gene. In each case, whatever change has made these killers independent of m is reversed by the mere process of mating. One of these strains has an approximate 20-fold increase in its content of the 1.7×10^6 dalton ds RNA. Another has no detectable ds RNA of any size.

. . . . . Drs. R. B. Wickner and M. J. Leibowitz

VI. POLYAMINES

The Search for Mutants of E. coli Defective in the Biosynthesis of the Polyamines

We have developed a method for the rapid screening of large numbers of cells for mutants defective in any reaction which produces CO_2. With this technique, we have isolated a mutant of *E. coli* that has a deficiency in adenosylmethionine decarboxylase, and hence is defective in spermidine biosynthesis.

. . . . . Drs. H. Tabor, C. W. Tabor, and E. W. Hafner

Genetic and Biochemical Studies of the First Step in Spermidine Biosynthesis in E. coli, Adenosylmethionine Synthetase

We have purified the adenosylmethionine synthetase of *E. coli* to homogeneity. This enzyme has wide significance in the total biochemistry of *E. coli*, since the adenosylmethionine not only serves as a precursor for polyamines, but serves as the methyl donor in methylation reactions. We have obtained a temperature sensitive enzyme in a mutant of *E. coli*, which suggests that the *metK* gene is the structural gene for this enzyme, and have carried out mapping experiments with this mutant.

. . . . . Drs. E. W. Hafner, H. Tabor, and C. W. Tabor

Eukaryotic (Yeast) Adenosylmethionine Decarboxylase

Adenosylmethionine decarboxylase of eukaryotes differs from that of prokaryotes in its requirement for putrescine. We have purified the enzyme from yeast and are carrying out studies to characterize the carbonyl cofactor.

. . . . . Drs. M. S. Cohn, C. W. Tabor, and H. Tabor

Studies on Glutathione and Glutathionylspermidine

We have continued to develop methods for the analysis of glutathione by automated anion exchange chromatography, and to study the biosynthesis and degradation of glutathione and glutathionylspermidine in *E. coli*.

. . . . . Drs. C. W. Tabor and H. Tabor

VII. EVOLUTION

Experiments on the origin of the genetic code have demonstrated substantial affinity with some discriminatory capacity between hydrophobic peptides and alkyl uracils. The complexes appear to form in large aggregates, which would favor their participation in primitive processes leading to the origin of life, according to our previously published theory.

. . . . . Drs. H. M. Chao and S. Black

VIII. TRAUMATIC SHOCK AND CELLULAR IMMUNITY

Experiments on the effect of histamine in burn, tourniquet, and endotoxin shock in mice suggested that endogenous histamine is not a lethal factor in burn and tourniquet shock as previously believed, but rather it appears to have a compensatory, beneficial effect.

Studies on the number and function of T and B cells from the spleens of normal and burned mice indicated that the decreased numbers and mitotic activity of T cells could play a role in the impaired cellular immunity after burns, but the same findings in B cells could not be critical in the development of normal humoral immunity in the absence of an overwhelming infection after thermal trauma.

In other experiments, various vasoactive hormones were studied on their effect upon swelling produced by a mild vacuum applied to the mouse tail. Compensatory mechanisms are such that inhibition of swelling results in most cases, even with capillary dilators.

. . . . . Drs. K. Markley and S. M. Rosenthal and Mrs. E. T. Smallman

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IX. MYCOBACTERIUM

The motion picture studies on the differentiation and maturation of mouse granulocytes was completed. A promyelocyte required six successive cell divisions in a period of 5 days to produce a total of 64 granulocytes. A "weaning" stage of 1-2 days was observed after the last cell division. Another 3-4 days was required for the cells to become the mature granulocytes.

A new technique for the quantitative estimation of the growth of granulocytes and macrophages has been developed. Endotoxin-treated (*in vivo*) mouse serum and endotoxin-treated (*in vivo*) mouse lung tissue conditioned medium showed strong stimulating effect on the growth of granulocytes in cultures.

Cytochemical studies on the so-called "novel cell" revealed that these cells were probably a type or a stage of macrophages.

Growth of *Mycobacterium lepraemurium* in cultures of macrophages obtained from various tissue sources, i.e., bone marrow, spleen, and blood leukocytes, was accomplished.

The effect of two anti-amebic drugs on murine leprosy was studied in mice. Both drugs, broxyquinoline and broxaldine, showed little activity.

. . . . . Dr. Y. T. Chang

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 23,140-18 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Biochemistry of Sulfur-Containing Compounds

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

PI: Simon Black, Ph.D. Biochemist LBP NIAMDD
and Chief, Section on Biochemistry of Amino Acids LBP NIAMDD

OTHER: Helen M. Chao, Ph.D. Staff Fellow LBP NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Biochemistry of Amino Acids

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.2	2.0	2.2

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

I. Glycyl tRNA synthetase regulation. A study of glycycl tRNA synthetase has revealed that two forms of this enzyme are interconverted in yeast cells as part of the operation of a regulatory mechanism that should be vital in governing the synthesis of proteins. The cyclic interconversion is now being studied *in vitro*.

II. Evolution. Evidence has been obtained that a number of 5'-substituted uracils have a substantial affinity and modest discriminating capacity for certain hydrophobic peptides. These results support the possibility of a direct template-peptide coding system such as we proposed earlier as a mechanism for the early evolution of life and origin of the genetic code.

PHS-6040
(12-75)

Project Description:

I. *Protein Synthesis.* The aminoacyl tRNA synthetases of all living organisms catalyze the following general reaction:

$$\text{amino acid} + \text{ATP} + \text{tRNA} \rightleftharpoons \text{aminoacyl tRNA} + \text{AMP} + \text{PP}.$$

We have found previously that within the yeast cell two forms of the glycyl tRNA synthetase co-exist, one being dependent on activator-like substances, whereas the other is active in the absence of any special additives. These two forms appear to be cyclically interconverted during growth as part of a regulatory mechanism. During the past year we have sought to demonstrate this interconversion of the two forms in cell-free extracts. A number of unique problems were encountered. Because of the cyclic process, each new batch of yeast grown for making enzyme extracts is different from all others, and reproducible procedures on an adequate scale have been difficult to develop. However, our most recent results indicate that the *in vitro* process will soon be under sufficient control for fruitful experimentation.

We plan to continue this study with the general objective of unraveling and illucidating the detailed components and operation of the mechanism by which the primary step of protein synthesis is regulated.

II. *Evolution.* Experiments designed to elucidate the origin of the genetic code have been further pursued. Five hydrophobic pentapeptides, representing the five amino acids of the uracil column of the genetic codon table, have been tested for their affinity for thirteen different alkyl uracil derivatives. Many of the affinities are presumed adequate for function in a primitive peptide-template translation process. The patterns of interaction are unique for each peptide, so that hypothetical templates of the bases would yield peptide products of markedly differing compositions, probably suitable for evolutionary selection. The complexes of peptide and base in many instances appear to form as part of large aggregates, which accords with the general theory on the origin of life that we have proposed, wherein initial processes occurred in a membrane-like film at an oil-water interface.

A recently completed project in this area involved the purification of an enzyme from *Erwinia herbicola* that catalyzes a phosphorylation of nucleosides by p-nitrophenylphosphate. It is hoped to use this enzyme for the preparation of nucleotides of "primitive codons," eventually to link these together as primitive nucleic acids. The enzyme has been obtained in a reasonable state of purity and characterized.

Publications:

Chao, H. M.: Nucleoside phosphotransferase from *Erwinia herbicola*, a new membrane-bound enzyme. J. Biol. Chem. 251: 2330-2333, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 23,230-26 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Chemotherapy of Mouse Leprosy

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

PI: Yao Teh Chang, M.D. Research Pharmacologist LBP NIAMDD

COOPERATING UNITS (if any)
Akira Yamagami, M.D., National Institute for Leprosy Research, Tokyo, Japan (Guest Worker, LBP, NIAMDD from October 1, 1975, to February 28, 1976)

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.0	1.2

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

The motion picture studies on the differentiation and maturation of mouse granulocytes was completed. A promyelocyte required six successive cell divisions in a period of 5 days to produce a total of 64 granulocytes. A "weanning" stage of 1-2 days was observed after the last cell division. Another 3-4 days was required for the cells to become the mature granulocytes.

A new technique for the quantitative estimation of the growth of granulocytes and macrophages has been developed. Endotoxin-treated (*in vivo*) mouse serum and endotoxin-treated (*in vivo*) mouse lung tissue conditioned medium showed strong stimulating effect on the growth of granulocytes in cultures.

Cytochemical studies on the so-called "novel cell" revealed that these cells were probably a type or a stage of macrophages.

Growth of *Mycobacterium lepraemurium* in cultures of macrophages obtained from various tissue sources, i.e., bone marrow, spleen, and blood leukocytes, was accomplished.

The effect of two anti-amebic drugs on murine leprosy was studied in mice. Both drugs, broxyquinoline and broxaldine, showed little activity.

Project Description:

Growth of Mouse Granulocytes. Investigators working in the leprosy field always wondered how little knowledge is available even on a fundamental question that a method for cultivation of *Mycobacterium lepraemurium* is still not possible. When we come to the field of leukemia, one is even surprised more by the fact that a method for the growth of normal leukocytes is still in its primitive stage. The fundamental question of how many mature cells can be produced by a promyelocyte and how many differential steps are required for such a process is still not answered. If one does not know how to grow the normal granulocytes, how can one deal with the abnormal cells, the leukemia cells.

In the past 5 years, we have been working on the growth of granulocytes in tissue cultures. A better medium has been worked out. A simple method for taking long-term, continuous motion pictures has been developed. This allowed us to follow various stages of development of the growth of granulocytes in cultures. The time schedule of differentiation and maturation of granulocytes is as follows:

Granulocytes apparently grew on top of a large macrophage, the natural feeder cell. A promyelocyte required 6 successive cell divisions in a period of 5 days to become the granulocytes. The average generation time of each cell division was approximately 20 hours (13.1 to 23.5 hours). A "weaning" stage of 1-2 days was followed in which the cells struggled violently to leave the feeder cell, and kept on shaking, jumping in the medium thereafter. It required another 3-4 days for these cells to reach the typical mature form, i.e., moved slowly toward various directions, yet remained more or less in the same location. Thus a period of 5 days mitotic differentiation and 6 days maturation is required for a promyelocyte to produce a total of 64 mature granulocytes.

Quantitative Estimation of Granulocytes. The current method for cultivation of granulocytes has three serious problems. First, granulocytes multiply for only a short time in the cultures. After 5-7 days most of the granulocytes disappeared, and marked growth of macrophages appeared at the same site forming a large colony. Evaluation of growth of granulocytes is based on enumeration of the macrophage colonies. Second, identification of cell type in a semi-gel culture system is poor. For half the time, one is not able to distinguish the mononuclear cells with granulocytes. Yet, the percentage of colonies of granulocytes, macrophages, or a mixture of granulocytes and macrophages, have been mentioned many times in the literature. Third, smaller colonies which are less than 50 cells are not included in the colony counting. Unfortunately, the number of such colonies are plenty, and they do contain many granulocytes.

Our method eliminates all these difficulties. The growth of granulocytes was made on conical plastic tubes without agar. The granulocytes were vibrated off the tube on a super mixer. The cells were collected, the numbers counted, and a cytocentrifuge smear was made. The smear was stained with Sudan black B and May-Grunwald-Giemsa, which differentiate the

granulocytes beautifully. The tubes were then placed in a 50° water bath for 10 minutes. The adherent macrophages were released by super mixer. A second cell count was made. In this way, a quantitative estimation of both granulocytes and macrophages was obtained. The importance of this method for future studies of granulopoiesis is evident.

Granulocyte Stimulating Agents. The effect of endotoxin-treated (*in vivo*) mouse serum was studied with our method. The stimulating effect was demonstrated even in a 1 : 1,000 dilution of the serum, compared to that of 1 : 150 dilution reported by others. A conditioned medium made by cultivation of lung tissue from mice which had received endotoxin previously also showed high stimulating activity.

Cytochemical Studies of "Novel Cells." A dendritic cell type has been considered as a "novel cell" type by other investigators since these cells do not show any properties of macrophages. We found that these cells (in cultures of spleen and lymph node cells) revealed strong activity of nonspecific esterase, PAS reaction, ferritin deposition, phagocytosis of *Mycobacterium lepraemurium*, and surface receptors for antibodies (sheep erythrocyte rosette formation). Since these features are the properties of macrophages, the novel cell was probably a type or a stage of macrophages.

Growth of M. lepraemurium in Various Sources of Macrophages. Dr. Akira Yamagami, a guest worker in this laboratory from the National Institute for Leprosy Research, Tokyo, Japan, has succeeded in the growth of *M. lepraemurium* in cultures of macrophages obtained from bone marrow, spleen, and blood leukocytes.

Chemotherapy of Murine Leprosy. The effect of two anti-amebic drugs on murine leprosy was studied in mice. Both drugs, broxyquinoline and broxaldine, showed little suppressive activity on the growth of murine leprosy.

Publications:

Chang, Y. T.: A simple method for copy of frames of a 16 mm motion picture. In Evans, V. J., Perry, V. P., and Vincent, M. M. (Eds.): Tissue Culture Association Manual. Rockville, Maryland, Tissue Culture Association, Issue 2, 1975, pp. 95-96.

Chang, Y. T.: Long-term time lapse photomicrography. In Evans, V. J., Perry, V. P., and Vincent, M. M. (Eds.): Tissue Culture Association Manual. Rockville, Maryland, Tissue Culture Association, Issue 2, 1975, pp. 99-102.

Chang, Y. T.: Low-magnification time lapse photomicrography. In Evans, V. J., Perry, V. P., and Vincent, M. M. (Eds.): Tissue Culture Association Manual. Rockville, Maryland, Tissue Culture Association, Issue 2, 1975, pp. 97-98.

Chang, Y. T., and Andersen, R. N.: Cultivation of mouse bone marrow cells: cytochemical studies of the granulocyte colony feeder cells. J. Reticuloendothel. Soc. 18: 34-43, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 23,580-13 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Protein Synthesis in *Escherichia coli*

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

PI: Anthony V. Furano, M.D. Medical Officer (Research) LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

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

We are currently investigating the following areas:

I. The mechanism and control of protein synthesis in *Escherichia coli in vitro* and *in vivo* with particular emphasis on the role and means of regulation of the elongation factor Tu.

II. The role of protein methylation in biological regulation.

PHS-6040
(12-75)

Project Description

I. *Protein Synthesis.* Work from the laboratory has established that the peptide chain elongation factor (EF) Tu is present at considerable molar excess over ribosomes. This finding which has recently been confirmed by reports from several other laboratories and contrasts with the results found for EF-G and EF-Ts which are present at a 1 : 1 molar ratio to ribosomes raised several questions which are currently being investigated: (1) Do all the molecules of EF-Tu have the same structure and function? (2) How does the cell coordinate the synthesis of EF-Tu, EF-G, and EF-Ts and ribosomes but still maintain the molar excess of EF-Tu?

Recent results from this laboratory bearing on the first question have shown that 75% of the EF-Tu in cell extracts is free of other macromolecules, whereas 10% is bound to EF-Ts. However, 10-15% of the Tu is reproducibly found in some kind of aggregated form, the nature of which is not clear and is currently under investigation. Tryptic peptide maps of the three forms of EF-Tu are indistinguishable but suggest that EF-Tu exhibits microheterogeneity. These data indicate that not all the molecules of EF-Tu are the same, a finding which may be related to the recent discovery by Nomura and coworkers that there are two genes for Tu in *E. coli*. In fact, work is now underway to compare the products of these two genes by tryptic-peptide maps.

Pertaining to this second question, we have recently shown that the relA gene affects the synthesis of EF-Tu and EF-G as it does the synthesis of stable RNA and ribosomal proteins, i.e., these elongation factors are under stringent control. In the course of these studies we have also shown that the synthesis of a great number of nonribosomal proteins are affected by expression of the relA allele. We measured the rates of synthesis of various individual proteins in growing relA$^+$ and relA$^-$ strains under conditions in which the two strains would have very different concentrations of the guanine nucleotide ppGpp. (RelA$^+$ synthesizes ppGpp under conditions of partial amino acid starvation; relA$^-$ cells cannot.) The data suggest that ppGpp inhibits or stimulates the transcription of many bacterial genes. Work on this phenomenon is continuing, and we have most recently shown that high levels of ppGpp stimulates the synthesis of some amino acid biosynthetic enzymes but not of others.

II. *Protein Methylation.* By use of our recently developed rapid and easy method for the analysis of methylated amino acids in proteins, we showed that in contrast to the rich array of methylated proteins in the various subcellular fractions of eukaryote, only *E. coli* 50 S ribosomal proteins contained methylated amino acids. However, recent evidence by others has shown that a rapidly metabolized methylated protein may be involved in the chemotactic response of bacteria. These findings have prompted new studies on our part to examine the question of reversible methylation of proteins in *E. coli*.

Publications

Furano, A. V.: Content of elongation factor Tu in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. 72: 4780-4784, 1975.

Furano, A. V.: The subcellular distribution and state of the elongation factor Tu in extracts of *Escherichia coli* B. Eur. J. Biochem. 64: 597-606, 1976.

Furano, A. V., and Wittel, F. P.: Effect of the relA gene on the synthesis of individual proteins *in vivo*. Cell, in press.

Furano, A. V., and Wittel, F. P.: Syntheses of elongation factors Tu and G are under stringent control in *Escherichia coli*. J. Biol. Chem. 251: 898-901, 1976.

Klagsbrun, M., and Furano, A. V.: Methylated amino acids in the proteins of bacterial and mammalian cells. Arch. Biochem. Biophys. 169: 529-539, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 23,630-11 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biology of Complex Carbohydrates

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

PI:	Victor Ginsburg, Ph.D.	Research Chemist	LBP NIAMDD
	and Chief, Section on Biochemistry		LBP NIAMDD
OTHER:	David A. Zopf, M.D.	Senior Staff Fellow	LBP NIAMDD
	Chao-Ming Tsai, Ph.D.	Staff Fellow	LBP NIAMDD

COOPERATING UNITS (If any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.7	0.3

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

Oligosaccharides will be isolated from natural sources. Certain oligosaccharides will be coupled to macromolecules in order to make antibodies specific for certain sugar sequences. The antibodies will be used in radioimmune assays for the occurrence of these sugar sequences in the glycolipids and glycoproteins of cell membranes and serum.

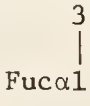
Waldenström macroglobulins and myeloma proteins will be screened for their ability to react with human erythrocytes. Those that do will be tested for their ability to react with various oligosaccharides coupled to polylysine in order to define their specificity.

PHS-6040
(12-75)

Project Description:

A cold agglutinin (McC) was isolated from serum of a patient with Waldenström's macroglobulinemia by affinity chromatography using temperature elution from a column of glutaraldehyde-fixed erythrocyte stroma mixed with celite. The agglutinin, which is an IgM kappa, reacts weakly with normal adult erythrocytes and strongly with cord cells, as well as with adult O_I cells, and, therefore, by definition has "anti-i" specificity. The reactivity of all cell types tested increased to approximately the same level after treatment of the cells with ficin. The purified cold agglutinin forms precipitin lines with polylysine conjugates containing oligosaccharides with the non-reducing sequence Galβ1-4GlcNAcβ1-3Gal.... Agglutination of erythrocytes at 4° is specifically inhibited by oligosaccharides with the same non-reducing sequence and by "paragloboside," a glycosphingolipid extracted from human erythrocyte membranes with the structure Galβ1-GlcNAcβ1-3Galβ1-4Glc-ceramide. In contrast, another cold agglutinin (Ma) which agglutinates adult erythrocytes in preference to cord blood erythrocytes and therefore has anti-I specificity, precipitates only with polylysine-oligosaccharide conjugates containing the non-reducing sequence Galβ1-4GlcNAcβ1-6Gal.... This structure occurs in adult erythrocyte membranes in glycosphingolipids containing ten monosaccharide residues. Ficin treatment produces little change in reactivity of adult cells with Ma. It has been observed previously that glycosphingolipids with four or fewer glycosyl residues tend to behave as cryptic receptors in adult cells in that they bind antibodies only after treatment of the cells with proteolytic enzymes, while the same molecules in fetal cells bind antibodies without proteolysis. These results suggest that the chain length of carbohydrates on surfaces of adult and fetal cells might explain the differential expression of receptors for "anti-I" and "anti-i" agglutinins on human erythrocytes.

The oligosaccharides of human milk vary with the blood type of the donor as the enzymes that synthesize them also synthesize the complex carbohydrates of cell surfaces. During the fractionation of milk from a donor with the genotype *seselele* [a non-secretor with a Le(a^-b^-) blood type], a fucose-containing pentasaccharide was isolated which was not previously detected in milk from donors possessing an *se* gene, an *Le* gene, or both. Its structure was determined to be Galβ1-3GlcNAcβ1-3Galβ1-4Glc.



Proposed Course:

Oligosaccharides will be isolated from natural sources. Certain oligosaccharides will be coupled to macromolecules in order to make antibodies specific for certain sugar sequences. The antibodies will be used in radioimmune assays for the occurrence of these sugar sequences in the glycolipids and glycoproteins of cell membranes and serum.

Waldenström macroglobulins and myeloma proteins will be screened for their ability to react with human erythrocytes. Those that do will be tested for their ability to react with various oligosaccharides coupled to polylysine in order to define their specificity.

Publications:

Ginsburg, V., Zopf, D. A., Yamashita, K., and Kobata, A.: Oligosaccharides of human milk. VII. Isolation of a new pentasaccharide, lacto-N-fucopentaose V. Arch. Biochem. Biophys., in press.

Joseph, K. C., and Gockerman, J. P.: Accumulation of glycolipids containing N-acetylglucosamine in erythrocyte stroma of patients with congenital dyserythropoietic anemia type II (HEMPAS). Biochem. Biophys. Res. Commun. 65: 146-175, 1975.

Tsai, C.-M., Zopf, D. A., Ginsburg, V., and Wistar, R., Jr.: A human cold agglutinin which binds lacto-N-neotetraose. J. Immunol., in press.

Zopf, D. A., Ginsburg, A., and Ginsburg, V.: Goat antibody directed against a human Le^b blood group hapten, lacto-N-difucohexaose I. J. Immunol. 115: 1525-1529, 1975.

Zopf, D. A., Tsai, C.-M., and Ginsburg, V.: Studies on the carbohydrate receptors of cold agglutinins using synthetic antigens. In Mohn, J. F. (Ed.): Human Blood Groups. Basel, Switzerland, S. Karger AG, in press.

Project No. Z01 AM 23,930-06 LBP
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1975 through June 30, 1976

Project Title: Role of Subunit Interactions in Enzyme Chemistry

Previous Serial Number: Z01 AM 23,930-06 LBP

Principal Investigator: Dr. Claude B. Klee

Other Investigators: None

Cooperating Units: Drs. Louis A. Cohen and Kenneth L. Kirk, Laboratory of Chemistry, and Dr. Peter McPhie, Laboratory of Biochemistry and Metabolism, National Institute of Arthritis, Metabolism, and Digestive Diseases

Man Years:

Total:	0.00
Professional:	0.00
Others:	0.00

Project Description: This project was terminated on January 19, 1974.

Publications:

Klee, C. B., Kirk, K. L., Cohen, L. A., and McPhie, P.: Histidine ammonia-lyase. The use of 4-fluorohistidine in identification of the rate-determining step. J. Biol. Chem. 250: 5033-5040, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 23,960-10 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cell Regulation by the Action of Pharmacodynamic Agents on the Cell Membrane

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

PI:	Leonard D. Kohn, M.D.	Medical Officer (Research)	LBP NIAMDD
	and Chief, Section on Biochemistry of Cell Regulation		LBP NIAMDD
OTHER:	Brian R. Mullin, M.D.	Senior Staff Fellow	LBP NIAMDD
	Salvatore M. Aloj, M.D.	Visiting Scientist	LBP NIAMDD

COOPERATING UNITS (If any)

Please see next page.

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Biochemistry of Cell Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.9	1.1

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

The structure of the thyrotropin receptor and the mechanism of action of thyrotropin have been shown to be analogous to that of cholera toxin, i.e., a critical role for gangliosides has been defined. This structure and mode of action appear to be applicable to the other glycoprotein hormones (human chorionic gonadotropin, luteinizing hormone, and follicle-stimulating hormone) and to effectors of cell function such as interferon.

Human adipocytes and adipocyte membranes can bind thyrotropin and its exophthalmogenic derivative (formed by partial pepsin digestion of thyrotropin). The binding is enhanced by γ globulin from patients with Graves' disease and exophthalmos. The two-factor hypothesis of exophthalmos which invokes an abnormally functional TSH receptor in retro-orbital tissue cells is thus applicable to humans. A soluble TSH binding component in the cytosol has been described.

The antigen responsible for inhibiting the migration of leucocytes from patients with Graves' disease, i.e., for the cellular hyperimmunity of these patients has been partially purified. Current evidence suggests that the antigen is thyroglobulin.

A C terminal procollagen peptidase activity has been defined.

COOPERATING UNITS

Peter H. Fishman, Ph.D., Developmental and Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland

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Project Description:

Objectives:

The thrust of work in this section is toward an understanding of the mechanisms regulating cell activity. Special emphasis is on studies concerning the action of pharmacodynamic agents on enzymes within the cell and in its membrane. Specifically, studies have been initiated which concern the mechanism of exophthalmos and Graves' disease, preventive therapeutic measures for these diseases, and hormone receptor function at a membrane level. These studies have been extended to the enzymatic mechanisms of membrane transport in bacterial and mammalian cells; to membrane structure; to enzymatic mechanisms in the biosynthesis of collagen and their relation to human collagen disease states; and to the structure and function of oxido reductases important to energetic regulation of cell functions.

Major Findings:

I. *Studies Concerning the Mechanism of Exophthalmos and of Thyrotropin Receptor Function.* We have suggested that malignant exophthalmos in humans is a two-factor disease caused by the simultaneous presence of thyrotropin (TSH) or a derivative of the TSH molecule with no thyroid-stimulating ability and by the presence of an abnormal or autoimmune gamma globulin. The TSH or TSH derivative served as the direct effector while the gamma globulin targeted this effector by increasing its binding to retro-orbital tissue. Our evidence which led to this hypothesis can be summarized as follows: [1] the thyrotropin molecule is exophthalmogenic; [2] the thyrotropin molecule can be fragmented by partial pepsin digestion to yield a derivative (EPF) with exophthalmogenic activity but no thyroid-stimulating ability; [3] this derivative (as well as TSH) can induce exophthalmos and the biochemical changes characteristic of exophthalmos in the guinea pig, a mammalian model of exophthalmos; [4] there is an exophthalmogenic factor in the sera of exophthalmic patients which is in the gamma globulin fraction of these sera and is not the long-acting thyroid stimulator (LATS); and [5] this gamma globulin from the sera of exophthalmic patients significantly increases the *in vitro* binding of TSH and EPF to retro-orbital tissue plasma membranes but not to thyroid plasma membranes.

The gamma globulin from malignant exophthalmos patients enhances TSH binding or the binding of its exophthalmogenic derivative to retro-orbital tissue plasma membranes by increasing the number of TSH receptors, by shifting the association constants of receptors to much lower levels, and by decreasing the negatively cooperative relationship amongst receptor sites. Most important in this regard, the gamma globulin does not modify the binding site or the receptor directly. Its action requires the presence of TSH or its exophthalmogenic derivative; hence, the gamma globulin must function either to modify the TSH or EPF molecule itself or to interact with the membrane only after TSH has been bound.

In previous work, the *in vitro* binding properties of thyrotropin receptors on both retro-orbital tissue and thyroid tissue plasma membranes were characterized and found to be effectively identical. Despite these functional

similarities, tryptic digestion of thyroid plasma membranes released a specific thyrotropin receptor 15,000 to 30,000 in molecular weight, whereas tryptic digestion of retro-orbital tissue membranes released a 75,000 to 150,000 molecular weight thyrotropin receptor. Since this structural difference might correlate with the different effects of exophthalmic gamma globulin on binding in these two membrane preparations, the receptors were further studied.

In studies reported this year we have shown the following:

(A) The tryptic fragment of the bovine thyroid TSH receptor has been purified. This purified 15,000 to 30,000 molecular weight receptor component is specific in its binding of ^{125}I-TSH. When binding is evaluated as a function of hormone concentration, a nonlinear Scatchard plot is measured which is analogous to that determined on the heterogeneous TSH receptor preparation produced by LIS solubilization. It has the same pH optima, hormone specificity, and salt inhibition phenomena as the LIS solubilized preparation untreated with trypsin. Disc gel electrophoresis of the purified receptor fragment shows a major protein and carbohydrate staining component with a molecular weight of 24,000.

The binding activity of the receptor fragment is lost when it is exposed to beads of neuraminidase-Sepharose or conconavalin A-Sepharose but is not lost when exposed to Sepharose alone, collagen-Sepharose preparations, or procollagen-Sepharose preparations. Consistent with these data, carbohydrate analyses indicate that the TSH receptor fragment preparation has a 30% carbohydrate and 10% sialic acid content.

An antiserum prepared against the TSH receptor fragment reacts not only with the immunogen but also with the heterogeneous LIS solubilized receptor preparation. The antiserum can precipitate soluble receptor activity. The antiserum also reacts against solubilized guinea pig retro-orbital tissue preparations.

(B) Our early experiments which used [^{3}H]TSH or [^{3}H]EPF and guinea pig Harderian gland plasma membranes could be questioned on the following point: the applicability of studies using membranes from a non-human source, the guinea pig Harderian gland. To obviate this point, studies on human retro-orbital tissue adipocytes were performed. ^{125}I-TSH binding to these adipocytes was significantly enhanced by the autoimmune gamma globulin as compared to normal gamma globulin at the same concentration. In contrast, control experiments using dog retro-orbital tissue adipocytes showed that ^{125}I-TSH could be bound but that there was no significant increase by the different gamma globulin preparations. In addition, both human and guinea pig adipocyte membranes were shown to have specific thyrotropin receptor sites, and the thyrotropin binding to human retro-orbital tissue adipocyte membranes was shown to be enhanced by gamma globulin from patients with exophthalmos. The findings are thus analogous to those obtained with guinea pig retro-orbital tissue membrane preparations despite the morphologic differences in the two tissues. The data allow our extrapolation of the "two-factor" mechanism for experimental exophthalmos to the human condition, although they are not a direct proof of its validity.

(C) Supernatant solutions from crude human and guinea pig membrane preparations have been shown to contain a thermolabile, nondialyzable inhibitor of thyrotropin binding which acts by forming a thyrotropin-inhibitor adduct rather than by directly interacting with the membrane receptors.

The existence of a soluble TSH binding factor is at least of experimental importance and may have significant physiological relevance. From the experimental point of view, binding of TSH to poorly washed membrane preparations might yield confusing data, since specific TSH-receptor binding would be hidden by the TSH interaction with a soluble factor which is not only not measured by the binding assay but competes with the receptor for the ^{125}I-TSH added to the incubations. From a physiological point of view, this binding component might play a significant role in transmitting the TSH message to the cell machinery; for example, it might play a role in the TSH effect on thyroglobulin biosynthesis. Such a factor would be analogous to those involved in the intracellular binding of estrogen and steroid hormones and more recently of thyroid hormone. The nature of this inhibitor and its relationship to both cell function and the TSH plasma membrane receptor is under active investigation.

(D) Gangliosides have been shown to inhibit ^{125}I-labeled thyrotropin binding to the thyrotropin receptors on bovine thyroid plasma membranes, on guinea pig retro-orbital tissue plasma membranes, and on human adipocyte membranes. This inhibition by gangliosides is critically altered by the number and location of the sialic acid residues within the ganglioside structure, the efficacy of inhibition having the following order: $G_{D1b} > G_{T1} > G_{M1} > G_{M2} = G_{M3} > G_{D1a}$. The inhibition results from the interaction of thyrotropin and gangliosides, rather than the interaction of membrane and gangliosides. Fluorescence studies show that the inhibition is associated with a distinct conformational change of the thyrotropin molecule and that the progression from a "noninhibitory conformation" to an "inhibitory conformation" parallels exactly the order of effectiveness in inhibiting ^{125}I-labeled thyrotropin binding. The ganglioside inhibition of ^{125}I-labeled thyrotropin binding is hormonally specific. The possibility that a ganglioside or ganglioside-like structure is a compound of the thyrotropin receptor is suggested by the finding that gangliosides more complex than N-acetylneuraminylgalactosylglucosylceramide are present in bovine thyroid membranes in much higher quantities than have been previously found in extraneural tissue. The finding that the B component of cholera toxin, which also interacts with gangliosides, has a peptide sequence in common with the β subunit of thyrotropin, suggests that thyrotropin and cholera toxin may be analogous in their mode of action on the membrane.

The present study raises the possibility that gangliosides are an important structural component of TSH receptors in all tissues or a structural analog of an "active site" important for binding in these membranes.

(E) The B chain of cholera toxin and the β subunits of thyrotropin, luteinizing hormone, human chorionic gonadotropin, and follicle-stimulating hormone have been shown to have a region of sequence analogy believed to correlate with their ability to bind to receptors on cell membranes. A possible sequence analogy is also defined in the α subunits of these glycoprotein

hormones and a region of the cholera toxin A_1 chain believed to be responsible for adenylate cyclase activation.

The sequence homologies between luteinizing hormone, human chorionic gonadotropin, TSH, and cholera toxin raise the possibility that luteinizing hormone and human chorionic gonadotropin also have a similar mechanism of receptor interaction but that each of these agents recognizes carbohydrate sequences distinct from those recognized by TSH or cholera toxin. Each target organ might thus have a receptor with a specific carbohydrate sequence on a ganglioside-like structure. The interaction of the appropriate hormone with this specific oligosaccharide would result in a unique conformational shift such that the α subunit would be placed in the position favored for adenylate cyclase activation in that particular target tissue. Interaction with the wrong hormone would result in a different conformation, an unfavorable position, and no second message. The predictions inherent in this hypothesis are being examined.

(F) Thyrotropin and cholera toxin have been shown to have an analogous mode of interaction with receptors on thyroid plasma membranes. Thus, in both the case of TSH and cholera toxin, the β or B subunit interacts with specific cell surface receptors having gangliosides or ganglioside-like oligosaccharides as part of their core structures. This binding induces a conformational change in the hormone or toxin which results in translocation of an "active" α or A subunit within the cell membrane and adenylate cyclase activation. The evidence which led to this hypothesis can be summarized as follows. Thyrotropin and cholera toxin both bind to gangliosides *in vitro*. The binding of both molecules to the gangliosides is critically affected by the number and location of sialic acid residues on the carbohydrate portion of the ganglioside molecule. The interaction of TSH and cholera toxin with gangliosides is associated with a change in their conformational states, which in the case of cholera toxin is believed to ultimately result in a dissociation of the toxin molecule. Gangliosides which bind best to TSH *in vitro*, i.e., G_{D1b}, G_{T1}, and G_{M1}, are present in thyroid plasma membranes in higher quantities than have been previously found in extraneural tissue. A sequence analogy can be demonstrated in the B chain of cholera toxin and the β subunit of TSH, both of which are believed to carry the primary determinants for interactions with receptors on cell membranes. Also, a sequence analogy exists between the α subunit of TSH and the cholera toxin A protein, the subunit believed to be associated with adenylate cyclase activation.

Implicit in this hypothesis is the requirement that G_{M1} or a G_{M1}-like structure in thyroid plasma membranes might under certain circumstances interact with either TSH or cholera toxin. Thus, cholera toxin should inhibit TSH binding to thyroid plasma membranes. Further, if TSH and cholera toxin do act through a common receptor containing a G_{M1} or a G_{M1}-like structure, their ability to stimulate adenylate cyclase activity should not be additive and G_{M1} should cause cholera toxin to have a detectable conformational change analogous to that which we demonstrated for TSH.

Studies performed in this laboratory have now shown that unlabeled cholera toxin inhibits ^{125}I-thyrotropin binding to throtropin receptors on thyroid

plasma membranes. Maximal inhibition by cholera toxin does not exceed 40%, whereas unlabeled thyrotropin completely inhibits ^{125}I-thyrotropin binding to these same membranes. Kinetic analyses of the binding data are compatible with the view that the cholera toxin decreases the number of receptor sites available to thyrotropin and that the mechanism by which the cholera toxin inhibits ^{125}I-thyrotropin binding to these receptor sites involves both competitive and noncompetitive elements.

Cholera toxin can stimulate adenylate cyclase activity in thyroid plasma membranes. Its effect is not additive with that of thyrotropin, and in fact cholera toxin can inhibit thyrotropin stimulation of the adenylate cyclase activity. NAD enhances cholera toxin stimulation of adenylate cyclase activity but has no enhancing effect on the stimulatory activity exhibited by thyrotropin.

The G_{M1} ganglioside in thyroid plasma membranes can be tritiated by treating membranes with galactose oxidase, followed by reduction with ^{3}H-labeled sodium borohydride. Cholera toxin at a concentration yielding maximal inhibition of thyrotropin binding to thyroid plasma membrane receptors prevents the labeling of G_{M1}. Fluorescence data indicate that the interaction between cholera toxin and G_{M1} results in a conformational change in the cholera toxin molecule. Analogous conformational alterations cannot be detected upon exposure of cholera toxin to 5-fold higher concentrations of G_{M2} or G_{M3}.

(G) Plasma membranes derived from a rat thyroid tumor which is unresponsive to thyrotropin but not to dibutyryl cyclic AMP are unable to bind ^{125}I-TSH. This inability to bind ^{125}I-TSH is not caused by the presence of membrane-associated proteases able to digest TSH. Although the supernatant phase of the thyroid tumor homogenates contains a soluble TSH binding component which inhibits ^{125}I-TSH binding to TSH receptors on plasma membranes, its level is the same as in homogenates of normal thyroid tissue, i.e., the absence of ^{125}I-TSH binding to thyroid tumor plasma membranes cannot be accounted for on the basis of an increased content of this soluble TSH binding component. Neither trypsin digestion nor solubilization with lithium diiodosalicylate are shown to expose thyrotropin receptors in a manner analogous to that seen in normal thyroid tissue. The absence of ^{125}I-TSH binding to the thyroid tumor has thus only been correlated with the absence in its plasma membranes of gangliosides (G_{D1b}, G_{T1}, and G_{M1}) which are capable of interacting with TSH and are present in normal thyroid plasma membranes. These results support the hypothesis that gangliosides or ganglioside-like molecules are a structural component of thyrotropin receptors on thyroid plasma membranes.

(H) Patients with Graves' disease and exophthalmos demonstrate delayed hypersensitivity to antigens present in extracts of certain normal human tissue, namely, thyroid gland and retro-orbital tissue. The delayed hypersensitivity can be assayed *in vitro* by quantitating the amount of a lymphokine, migration inhibition factor (MIF), which is produced within the T cells of patients with Graves' disease and exophthalmos and which is exposed to these antigens. A partial purification has been described for the retro-orbital tissue antigen which is responsible for the positive leucocyte migration inhibition factor assay exhibited by the sensitized lymphocytes of these patients.

The purified retro-orbital tissue antigen preparation demonstrates a 50- to 150-fold higher specific activity over crude homogenates in its ability to act as an antigen in the MIF assay of exophthalmic patients. Immunodiffusion, ultracentrifugation, and disc electrophoretic data indicate that the purified preparation obtained from *normal* retro-orbital tissue contains thyroglobulin or a derivative of thyroglobulin; immunofluorescence studies localize the antithyroglobulin reactive material to the plasma membranes of extraocular muscle fibers of normal individuals. On the basis of these data we conclude that thyroglobulin or a derivative of the thyroglobulin molecule is present in the retro-orbital tissue of normal individuals. Since we show that thyroglobulin purified from normal human thyroid glands and the purified retro-orbital tissue preparation are nearly equivalent as antigens in the MIF assay of exophthalmic patients, we conclude that thyroglobulin or an antigenic component of the thyroglobulin molecule is one of the antigens to which patients with Graves' disease and exophthalmos demonstrate delayed hypersensitivity.

II. *Studies on the Mechanism of Action of Interferon.* Cholera toxin added together with interferon inhibited the establishment of antiviral activity in mouse L-cells. Cholera toxin has no effect on antiviral activity established before its addition; however, treatment of cells with the toxin before addition of interferon also inhibited the establishment of antiviral activity.

The presumption of these experiments was that by interacting with gangliosides or ganglioside-like oligosaccharides on the L-cell membrane ($G_{M1} > G_{M2} > G_{M3}$), the cholera toxin might either directly or indirectly interfere with the gangliosides ($G_{T1} = G_{M2} > G_{M1a} > D_{M1}$) or ganglioside-like oligosaccharide structures believed important to interferon interactions with its cell membrane receptors. Direct *in vitro* binding experiments already indicate that this presumption is valid but have not defined whether the cholera toxin acts at the same receptor site as interferon, or at a distal or separate receptor site, and that this interaction results in a secondary change in the state of the membrane which prevents the interferon from establishing its antiviral effect.

In this regard, the common sequence region on the B subunit of cholera toxin and TSH β subunit become interesting in regard to the structure of interferon itself. If the presumption is correct that this sequence analogy is critical to interactions with ganglioside or ganglioside-like oligosaccharides on the cell membrane, one might anticipate an analogous sequence on interferon and even an analogous molecular structure on interferon to that of cholera toxin or TSH. This possibility is of special interest given the current problems with structural studies of interferon--both in terms of defining the determinants on the interferon molecule important to receptor interaction and in defining the determinants important to its antiviral action.

It is possible that the mechanism of action of cholera toxin may be less specific. Interferon could act simply by altering the cell surface and thereby induce changes which result in an intracellular antiviral state with several membrane-associated viral functions altered. This might explain why some reports have suggested that interferon primarily inhibits viral transcription; others, viral translation; and still others, egress of the virus

from the infected cell. Cholera toxin by binding to sites on the plasma membrane may prevent induction by interferon of cell surface generations which are necessary for the establishment of antiviral activity.

Since the mechanism of action of cholera toxin is so well understood, the present findings may help to elucidate the early stages of the mechanism of interferon action.

III. *Studies Concerning the Biosynthesis of Collagen.* A procollagen peptidase activity for the C terminal extension peptide of type I procollagen has been defined. Purification of this activity is being accomplished in collaboration with Dr. John H. Fessler, Molecular Biology Institute, University of California at Los Angeles.

IV. *Studies Concerning Membrane Structure and Function.* An improved purification procedure (using hydrophobic chromatography and antibody-Sepharose columns) has been worked out for D-lactic dehydrogenase.

V. *Studies Concerning Centrifugation.* As a cooperating unit, centrifugation studies were performed for the following individuals in this Institute: Paul Torrence, Allen N. Schechter, and Edith W. Miles; and for the following individuals outside of this Institute: Thressa C. Stadtman (National Heart and Lung Institute) and Othmar Gabriel (Georgetown University).

Significance to Biomedical Research and the Progress of the Institute:

The research is aimed at evaluating enzymatic mechanisms by which cellular activities are regulated by pharmacodynamic agents. The possibility that unregulated biosynthetic or degradative processes may convert TSH into EPF active material has been established, and a mechanism for human exophthalmos has been proposed on the basis of these data. A diagnostic method has been developed which predicts Graves' disease patients who will get exophthalmos during therapy and a therapeutic program to treat these patients is being tested. Our studies of type I and type III procollagen and procollagen peptidase in regard to collagen biosynthesis have become important in understanding fetal development and aging, as well as the pathogenesis of several connective tissue diseases. Diagnostic applications of our immunoassay procedures have been initiated in regard to the detection of nonafflicted carriers of several connective tissue diseases. Studies concerning the mechanism of action of interferon should advance procedures for its large scale purification as well as our knowledge of its ability to block viral infections.

Proposed Course:

Studies of the TSH-EPF relationship will be continued in an attempt to examine the mechanism by which EPF activity is generated in humans *in vivo*. The proposed diagnostic mechanism for human exophthalmos will be further tested as will the therapeutic program. The structure of glycoprotein hormone receptors and the mechanism of action of glycoprotein hormones will be further clarified. The mechanism of procollagen peptidase formation and control will be related to normal and disease states. The biosynthesis of

type I procollagen and type III procollagen will be studied. The enzymatic mechanism of active transport in bacteria will be further defined, and studies on thyroid cell transport will be initiated. The mechanism of interferon interaction with cell membranes will be further defined.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,020-13 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies on the Chemical and Physiological Properties of the Surface of *E. coli*

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

PI:	Loretta Leive, Ph.D.	Research Biologist	LBP, NIAMDD
OTHER:	William G. Coleman, Jr., Ph.D.	Staff Fellow	LBP, NIAMDD

COOPERATING UNITS (if any)
Dr. Shlomo Rottem, The Hebrew University, Jerusalem, Israel (formerly Division of Blood and Blood Products, Bureau of Biologics, National Institutes of Health)

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	2.0	0.7

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

The structure and assembly of the outer membrane of *E. coli* is being studied. The insertion of newly-synthesized lipopolysaccharide occurs at 20-50 insertion points in this membrane, from which it spreads throughout the membrane.

Studies using electron spin resonance techniques to analyze the fluidity of this membrane have shown that it is very rigid, relative to other membranes, and that the rigidity is reduced if either the length of the lipopolysaccharide carbohydrate moiety, or the absolute amount of lipopolysaccharide, is reduced. These results indicate that lipopolysaccharide is directly or indirectly responsible for the rigidity of this membrane.

A new transducing phage has been characterized in the *E. coli* strain, 0111:B4. The relationship of this phage to other known transducing phages is being studied.

PHS-6040
(12-75)

Project Description:

I. *Structure and Assembly of E. coli Membranes.* The surface of *E. coli* consists of an inner or cytoplasmic membrane, a rigid cell wall, and an outer membrane. The outer membrane is relatively simple in composition, is assembled from molecules of protein, lipid, and lipopolysaccharide (LPS) made elsewhere in the cell, and therefore serves as a good model for studying membrane structure and assembly.

Previous work from this laboratory indicated that there are about 10-50 insertion points in the outer membrane into which newly synthesized LPS is inserted, and from which it moves elsewhere in the membrane. Current experiments have used density labeling of such membranes to indicate that, after an initial period, in the outer membrane, the LPS becomes immobilized into "domains" that do not mix with subsequently incorporated LPS. The size and nature of these "domains" are being analyzed.

The outer membrane appears by several criteria to have true membrane (lipid-protein bilayer) character. However, several reports have suggested that lipid mobility in this membrane may be much more restricted than in the cytoplasmic membrane of these and other cells. To determine whether the LPS of this membrane affects the mobility, electron spin resonance spectral analysis was performed. Outer membrane preparations containing LPS with a long polysaccharide chain were much less fluid than preparations containing LPS with a short polysaccharide chain, and treatment with EDTA to reduce LPS content resulted in increased mobility. These results indicate that LPS, and especially its polysaccharide moiety, directly or indirectly causes the restricted mobility of the lipid hydrocarbon chains in the outer membrane.

II. *Physiology and Genetics of an E. coli Strain Important in Studies of Lipopolysaccharide and Membrane Assembly.* *E. coli* O111:B4 is of great interest both because it is the most common of the *E. coli* strains that are enteropathogenic for infants and because it has a very well-characterized LPS with a very long polysaccharide chain. For the latter reason it has been used for studies of the outer membrane in several laboratories. Such work has been hindered because standard genetic manipulations have been heretofore impossible in this strain. We have now worked out procedures for conjugation involving production of unstable heterogenotes (stable recombinants cannot be formed) and have derived substrains which can be transduced. We are currently mapping a large number of loci and comparing them to their positions in *E. coli* K12.

E. coli O111:B4 has been occasionally reported in the literature to carry a lysogenic phage, or a colicin, or both. We have now characterized a particle carried by our strain of *E. coli* O111:B4. This particle has the morphological characteristics, on electron microscopy, of the well-known transducing phage P1, but differs from it in antigenic specificity. It cannot plate well on any strain yet tested, but shows very high efficiency of generalized transduction into many strains, including *E. coli* K12. It therefore manifests the characteristics of a defective transducing phage. It is of interest because it may transduce a smaller portion of the genome than phage in current use and therefore may enable more precise fine-structure mapping.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,120-05 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies in Traumatic Shock and Cellular Immunity

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

PI:	Kehl Markley, M.D.	Medical Director (USPHS)	LBP NIAMDD
OTHER:	Sanford M. Rosenthal, M.D.	Scientist Emeritus	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.1	1.3	1.8

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

Experiments on the effect of histamine in burn, tourniquet, and endotoxin shock in mice suggested that endogenous histamine is not a lethal factor in burn and tourniquet shock, but rather it appears to have a compensatory, beneficial effect.

Studies on the number and function of T and B cells from the spleens of normal and burned mice indicated that the decreased numbers and mitotic activity of T cells could play a role in the impaired cellular immunity after burns, but the same findings in B cells could not be critical in the development of normal humoral immunity in the absence of an overwhelming infection after thermal trauma.

In other experiments, various vasoactive hormones were studied on their effect upon swelling produced by a mild vacuum applied to the mouse tail. Compensatory mechanisms are such that inhibition of swelling results in most cases, even with capillary dilators.

PHS-6040
(12-75)

Project Description

Objectives:

Our objectives are to (I) study the function of the lymphocyte and macrophage in traumatic shock of mice; (II) study the role of histamine in traumatic shock; (III) study the influence of hormones on local swelling of the mouse tail by decreased local atmospheric pressure; and (IV) study the nephrotoxic action of spermine.

Methods Employed:

(I) Burn trauma was produced by immersing NIH female mice to the axilla (2/3 body surface area) in water at 70° for 7 seconds. Afterwards the mice were treated with 3 ml 0.85% NaCl subcutaneously to prevent death. Lymphocytes from normal and burned mice were obtained from their spleens, and T and B cells were separated on a nylon wool column. Mitoses of these cells were studied by the incorporation of [^{3}H]thymidine into DNA. Mitogenesis of T cells was stimulated by PHA (Difco) and by a purified preparation made by us, while mitogenesis of B cells was stimulated by *E. coli* lipopolysaccharide. Plasma and serum were collected from normal and burned mice.

(II) Burn shock was produced in NIH female mice by the method described above, while tourniquet shock was produced by placing rubber bands in the inguinal region of both hindlegs for 75-90 minutes. Endotoxin shock was produced by intraperitoneal injection of 0.5 mg *E. coli* lipopolysaccharide. Free histamine release was measured in serum and urine using the enzymatic isotopic procedure of Beaven *et al.* Compound 48/80 and the antagonists of the H_1- or H_2-histamine receptors (diphenhydramine and burimamide) were drugs used to determine the role of histamine in shock. Mortality studies were performed to test the efficacy of various drugs in shock.

(III) Measurement of the rate of movement of fluid from the capillaries into the extravascular space was accomplished by applying a mild vacuum (-40 to -80 mm Hg) to the tail of the mouse and measuring the volume before and after decreased atmospheric pressure.

(IV) Various possible antagonists to the nephrotoxic action of spermine were injected into mice to elucidate the role of spermine in renal physiology and disease.

Major Findings:

(I) There was a significant decrease ($P < 0.05$) in the total number of spleen cells, as well as T and B cells, for 2 to 3 days after burning with a rapid return to normal and a subsequent rise above normal at 14 and 21 days postburn ($P < 0.05$). In tests for function, burned mice had a significant decrease in mitotic activity of both T and B cells in 74% and 56% of the experiments, respectively, during the 21-day postburn period. When mitosis of T cells was stimulated by the addition of phytohemagglutinin (Difco, PHA-P) to the culture, the response of burned mice was normal in 72% of the experiments performed throughout the 3-week postburn period. On the other hand,

when the T cells were stimulated by a purified preparation of phytohemagglutinin, the response of burned mice was markedly reduced in 63% of the experiments during the same time period. With mitogenic stimulation of B cells by lipopolysaccharide, the response was less than normal in 74% of the experiments.

(II) In burn shock, serum histamine rose significantly after injury, but there was no correlation between increased serum histamine and high mortality as a consequence of several therapy regimens. For example, after treatment with histamine or compound 48/80 before burning, there was a rise of serum histamine, yet shock mortality fell significantly. Although separate administration of antagonists of H_1- or H_2-histamine receptors had no effect on mortality, pretreatment with both diphenhydramine and burimamide significantly increased shock mortality. In tourniquet shock, serum histamine rose significantly, and treatment with both antagonists before trauma produced a significant elevation of shock mortality. In endotoxin shock, prior treatment with one or both drugs did not change mortality.

(III) In the study of the effect of vasoactive hormones on the swelling produced by vacuum applied to the mouse tail, compensatory mechanisms produced an inhibition of swelling in most cases, even with capillary dilators.

Significance to Biomedical Research and the Program of the Institute:

These studies aid in the understanding of the mechanism of impaired cellular immunity after trauma and the regulation of transvascular fluid movement after a vacuum. In the histamine experiments, the results challenge the old concept that histamine could produce the effects of traumatic shock, since our data indicate that histamine could have a compensatory, beneficial effect in traumatic shock.

Proposed Course:

(I) To study the function of lymphocytes and macrophages in cellular immunity after burn and tourniquet trauma; (II) to study the function of lymphocytes in gnotobiotic mice; (III) to study factors that influence phagocytosis and intracellular killing of bacteria in macrophages; and (IV) to study the nephrotoxic action of spermine.

Publications:

Markley, K., Horakova, Z., Smallman, E. T., and Beaven, M. A.: The role of histamine in burn, tourniquet and endotoxin shock in mice. Eur. J. Pharmacol. 33: 255-265, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,140-10 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Chemistry and Mechanism of Pyridoxal Phosphate Enzymes

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

PI: Edith W. Miles, Ph.D. Research Chemist LBP NIAMDD

COOPERATING UNITS (if any)

None

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Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

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

The tryptophan synthase $\alpha_2\beta_2$ multienzyme complex is formed by the association of two α subunits and one β_2 subunit. The chemical nature of the subunit interaction sites, of the active site, and the factors influencing subunit association are being studied. The cofactor, pyridoxal 5'-phosphate and active site sulfhydryl and histidyl residues in the β_2 subunit are found to become partially shielded from the aqueous environment in the $\alpha_2\beta_2$ complex. Subunit association is favored by salts and by the cofactor, pyridoxal 5'-phosphate.

The substrate analog, *trans*-L-2-amino-4-methoxy-3-butenoic acid irreversibly inactivates the β_2 subunit by reacting with an active site nucleophilic group. Tryptophan synthase catalyzes two new pyridoxal 5'-phosphate dependent reactions in which β,γ-unsaturated amino acids are converted to saturated α-keto acids.

PHS-6040
(12-75)

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. The tryptophan synthase $\alpha_2\beta_2$ complex is the main enzyme under study. Since it is a multienzyme system in which one of the two protein species (the β_2 subunit) is a pyridoxal-P enzyme, it is a useful system for studying the effects of subunit interaction on the catalytic activities of the two different subunits and on the environment of amino acid residues in the active sites and interaction sites of the α and β_2 subunits.

Methods Employed:

Crystalline α and β_2 subunits and $\alpha_2\beta_2$ complex of tryptophan synthase have been prepared and assayed by methods previously developed in this laboratory. Absorbance spectra and difference absorbance spectra were made with a Cary 118 spectrophotometer. Circular dichroism spectra and fluorescence spectra were also made. Chemical modification of histidyl residues by diethylpyrocarbonate and sulfhydryl residues by dithiodinitrobenzoic acid have been carried out. Identification of α-hydroxy acids utilized gas-liquid chromatography, chemical ionization mass spectrometry, paper electrophoresis, and paper chromatography.

Major Findings:

The active site of the β_2 subunit has a different environment after the β_2 subunit has associated with the α subunit to form an $\alpha_2\beta_2$ complex. Essential histidyl and sulfhydryl groups in the active site of the β_2 subunit can be modified in the free native β_2 subunit but not in the native $\alpha_2\beta_2$ complex. Pyridoxal 5'-phosphate, the cofactor of the β_2 subunit, binds reversibly to the β_2 subunit but irreversibly to the $\alpha_2\beta_2$ complex.

Other features of the subunit interaction sites are being studied by determining factors necessary for subunit association. Low concentrations of NaCl promote subunit association, presumably by neutralizing the mutually repulsive ionized groups in the interaction sites of the α and β_2 subunits.

The substrate analog, *trans*-L-2-amino-4-methoxy-3-butenoic acid, irreversibly inactivates the β_2 subunit by reacting with an active site nucleophilic group. This substrate analog and 2-amino-3-butenoic acid are converted by the $\alpha_2\beta_2$ complex to corresponding saturated α-keto acids. These new reactions of tryptophan synthetase are important because they have been previously postulated as intermediate steps in pyridoxal phosphate enzyme catalyzed reactions of β or γ substituted amino acids. The demonstration of the tryptophan synthetase catalyzed reactions of β,γ-unsaturated amino acids demonstrates that these amino acids can be intermediates in reactions catalyzed by pyridoxal phosphate enzymes.

Publications:

Miles, E. W.: A new type of pyridoxal-P enzyme catalyzed reaction: the conversion of β,γ-unsaturated amino acids to saturated α-keto acids by tryptophan synthase. Biochem. Biophys. Res. Commun. 66: 94-102, 1975.

Miles, E. W.: Effects of modification of the β_2 subunit and of the $\alpha_2\beta_2$ complex of tryptophan synthase by α-cyanoglycine, a substrate analog. Biochem. Biophys. Res. Commun. 64: 248-255, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,260-10 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Enzymatic Studies of Nucleic Acid Metabolism

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

PI:	Nancy G. Nossal, Ph.D.	Research Chemist	LBP, NIAMDD
OTHER:	E. Bruce Konrad, Ph.D.	Staff Fellow	LBP, NIAMDD

COOPERATING UNITS (if any)

Frances D. Gillin, Ph.D., NIH Fellow and Guest Worker, LBP, NIAMDD, to 12/6/75

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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.5	0.3

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

Replication of T4 DNA *in vivo* requires several proteins encoded by the phage DNA including the gene 43 DNA polymerase, the gene 32 DNA unwinding protein, the gene 44 and 62 DNA-dependent ATPase, the gene 45 protein, and the gene 41 single-stranded DNA requiring nucleotidase. We have purified these proteins and have shown that they stimulate DNA synthesis *in vitro* with the T4 DNA polymerase with both native and single-stranded templates. We are presently studying the mechanism by which chains are initiated with circular single-stranded templates with these proteins, as well as mechanisms by which they accelerate the elongation of the primer.

Using a new screening procedure, we have identified a temperature-sensitive *E. coli* mutant (mut 3) with a high mutation frequency (mutator) which maps at or very close to the pol C locus encoding DNA polymerase III, as well as a second mutation which greatly enhances the mutator activity of mut 3 and of mutants in pol C. We plan to search for additional mutants of these types and to determine the role of the proteins affected by these mutations.

PHS-6040

Project Description:

Objectives:

To determine the characteristics and functions of enzymes which are involved in the synthesis, modification, and degradation of DNA.

Major Findings and Proposed Course:

I. Proteins Required for T4 Bacteriophage DNA Synthesis. We are using *E. coli* infected with T4 bacteriophage as a system in which to identify and characterize the reactions catalyzed by enzymes required for DNA replication, since this phage appears to induce the synthesis of the enzymes required for its own DNA synthesis, rather than depending on host proteins. Phage with mutations in genes coding for many of these enzymes are available due to the work of Edgar, Epstein, and their collaborators, and phage infected bacteria can be grown in quantities allowing purification of sufficient enzyme for biochemical and physical studies. The work has been facilitated by a regulatory phage mutant characterized by Wiberg which produces high levels of several of these enzymes.

Genetic studies by Edgar and Epstein *et al.* have shown that the proteins encoded by the phage genes 32, 41, 43, 44, 45, and 62 are required for DNA synthesis. Many other phage proteins are required for the synthesis of DNA precursors, or are involved in controlling the timing or extent of viral DNA synthesis. We have previously developed procedures for the purification to apparent homogeneity of the gene 32 DNA unwinding protein, the gene 43 DNA polymerase which also has 3' to 5' exonuclease activity, the complex of the gene 44 and gene 62 proteins which is a DNA-dependent ATPase (and dATPase), and the gene 45 protein which stimulates the ATPase activity of the 44-62 protein. The gene 45 protein is also required for late mRNA transcription (Geiduschek) and binds specifically to the T4 phage modified form of the host RNA polymerase (Ratner).

During the past year we have substantially improved the complementation assay used to detect the gene 41, 44/62, and 45 proteins, which is based on the ability of these proteins to stimulate DNA synthesis using an endogenous template in a crude lysate of cells infected with phage defective in that protein. The infected cells are now harvested and washed in one step by centrifugation through sucrose and are then lyzed after treatment with lysozyme by repeated freeze-thaw cycles in the absence of detergent.

Using this assay, we have purified the gene 41 protein and have shown that it is a nucleotide triphosphatase which requires single-stranded DNA for activity. When all of the ribo- and deoxynucleoside triphosphates are present together with single-stranded T7 DNA, the 41 protein hydrolyzes rGTP and rATP at similar rates to their respective diphosphates; dGTP and dATP at about half this rate; and dCTP and dTTP still more slowly. At the same concentration (of nucleotide equivalents) single-stranded DNA from phage T7, T4, and ϕX174 are equally effective in stimulating this reaction. Native duplex T7 or T4 DNA is less than one-tenth as active. The 41 nucleotidase

activity is not altered by the addition of T4 DNA polymerase and the gene 44/62 and 45 proteins under conditions where the single-stranded DNA serves as a template for DNA synthesis with these enzymes.

DNA synthesis using nicked duplex DNA templates at moderate salt concentrations (50 mM) requires the gene 32, 44/62, and 45 proteins in addition to the T4 DNA polymerase. We have not yet been able to demonstrate a need for the 41 protein with these templates.

Although the T4 DNA polymerase by itself can copy linear single-stranded templates, using the 3' OH end of the template as a primer, this synthesis is greatly stimulated by the gene 32, 44/62, and 45 proteins. The effect of these proteins is most pronounced with low concentrations of polymerase. We also find a modest stimulation (2-fold) of this synthesis by the 41 protein.

We have used single-stranded circular DNA templates (fd or $\phi\chi174$) in order to study the steps required to initiate the synthesis of a new chain. At high concentrations (40 µg/ml) the T4 DNA polymerase by itself will make a complete copy of these templates. The $\phi\chi$ product can be sealed to form duplex circular molecules by either the *E. coli* or T4 DNA ligase. The duplex circles have been characterized by sedimentation in alkaline and neutral sucrose with high salt, and by equilibrium density gradient centrifugation in ethidium-bromide-CsCl. A similar reaction by *E. coli* DNA polymerase I was shown by Goulian and Kornberg to be facilitated by the addition of oligonucleotides which served as primers. In order to remove ribo- or deoxyoligonucleotides which might be present in the template, we have sedimented the $\phi\chi$ DNA through alkaline sucrose, treated it with alkali under conditions hydrolyzing RNA, and incubated it with DNA exonucleases III and the 3' to 5' exonuclease of the L88 mutator T4 DNA polymerase (after heating and quick cooling the DNA) under conditions where exogeneously added oligonucleotides are hydrolyzed. None of these treatments diminishes the copying of the $\phi\chi$ DNA by T4 polymerase. Experiments are in progress to determine whether oligonucleotides are present in the enzyme or in other components of the reaction mixture which could serve as a primer, or whether there is *de novo* chain initiation by the enzyme. The latter seems unlikely since it has not been found with other DNA polymerases, or with the T4 enzyme using linear single-stranded templates.

At low DNA polymerase concentrations, the copying of circular templates is greatly stimulated by the 44/62, 45, 41, and 32 proteins. Although short product chains can be isolated at very short times or after incubation at low temperatures, the majority of the product molecules are close to full length, even when some template molecules have not been copied (as shown by sedimentation in high salt neutral sucrose gradients). We do not observe the absolute requirement for ribotriphosphates and 41 protein or the synthesis of product chains longer than the template which were demonstrated by Alberts and his coworkers with single-stranded circular fd DNA. Our inability to show a strong requirement for rXTP and 41 protein may be related to the fact that we get synthesis on circular templates with our preparation of T4 polymerase alone.

During the next year we will concentrate on determining how chains are initiated with circular templates. We will also investigate the mechanism

by which the 32, 44/62, and 45 proteins facilitate the elongation reaction. We have previously characterized mutant T4 DNA polymerases which differ from the wild-type enzyme in their affinity for DNA, their ability to discriminate against incorrect nucleotides, and in their capacity for strand displacement during the copying of duplex templates. Preliminary studies suggest the mutant polymerases also differ in some respects in their interaction with the 32, 44/62, and 45 proteins, and thus may prove useful in elucidating the role of these other proteins.

II. Isolation of Mutants Controlling Mutation Frequencies in E. coli. The fidelity of DNA replication is remarkably high. It is higher than would be predicted from *in vitro* studies of DNA polymerases. For example, in *E. coli*, an organism in which DNA replication has been extensively studied, the *in vivo* mutation rate may be two orders of magnitude lower than *in vitro* studies on one of the polymerases of this organism would indicate. This suggests that there may be mechanisms in the cell which can correct replication errors made by the polymerase. We have initiated a large scale search for conditionally lethal mutants of *E. coli* which enhance the frequency of replication errors (mutator mutants), since these may be useful in indicating which elements of the replication machinery are involved in maintaining their fidelity. Prior to this work, no large scale search for mutator mutations had been made, and only four mutator loci had been identified. None of these were conditionally lethal.

In order to accomplish such a search, we developed a method which permits large numbers of mutagenized colonies to be screened for the mutator trait. This method is as follows: Strains which are incapable of growth when lactose is the carbon and energy source (lac^-) are mutagenized and plated on lactose tetrazolium indicator plates. On those plates, lac^- colonies are red; however, lac^+ mutants which arise within a lac^- colony appear as white papillae on the colony's surface. The number of papillae is roughly proportional to the frequency of lac^+ mutants. Thus colonies with a high mutation rate can be identified. Conventional methods can then be used to confirm that a mutator mutation is present. Using this method, we have examined 1 million colonies and recovered 1,500 mutator strains. These have been screened for temperature sensitivity, since this sensitivity permits the physiology of the mutant to be more easily studied. Also if a mutator mutation affects DNA replication, one might expect that it could occur as a conditionally lethal, since replication is an essential process.

The result of this screening indicates that temperature-sensitive conditional lethal strains are very rare, since only one such strain was recovered. Preliminary study indicates that this mutant, mut 3, maps very near pol C, the locus which codes for polymerase III, the polymerase which may be responsible for most of *E. coli* DNA replication. However, it may constitute a new locus, since *in vitro* studies indicate that level of polymerase III in mut 3 is normal. DNA synthesis stops gradually after shift to high temperature with this mutant.

One interpretation of our finding that conditionally lethal mutator mutations are quite rare is that there may be a mechanism which corrects errors made during DNA replication, and thus masks all or most of the errors which

might be made by mutator mutations in the DNA replication machinery. If this is so, then inactivating this correction mechanism (by mutation, for example) ought to unmask replication errors made by, for example, a mutant DNA polymerase III and thereby enhance their activity as mutators. We have found at least one mutator which by itself is relatively weak but which becomes a strong mutator when combined with either mut 3 or pol C. Further genetic and biochemical experiments will be required to determine if it is in a correction mechanism.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of enzymes controlling the extent and fidelity of DNA replication is important to an understanding of the control of DNA replication and transcription in both normal and viral infected cells.

Publications:

Gillin, F. D., and Nossal, N. G.: T4 DNA polymerase has a lower apparent K_m for deoxynucleoside triphosphates complementary rather than noncomplementary to the template. Biochem. Biophys. Res. Commun. 64: 457-464, 1975.

Gillin, F. D., and Nossal, N. G.: Control of mutation frequency by bacteriophage T4 DNA polymerase. I. The CB120 antimutator polymerase is defective in strand displacement. J. Biol. Chem., in press.

Gillin, F. D., and Nossal, N. G.: Control of mutation frequency by bacteriophage T4 DNA polymerase. II. Accuracy of nucleotide selection by the L88 mutator, CB120 antimutator, and wild-type T4 DNA polymerases. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,510-02 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Phosphatidylserine Synthetase Mutants of *Escherichia coli*

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

PI: Christian R. H. Raetz, M.D., Ph.D.

Research Associate in Pharmacology NIGMS

Guest Worker LBP NIAMDD

COOPERATING UNITS (if any)

None

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NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

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

Mutants of *Escherichia coli* defective in CDP-diglyceride : L-serine phosphatidyltransferase (phosphatidylserine synthetase) can be isolated by a rapid autoradiographic screening assay described previously (Raetz, C. R. H., *Proc. Nat. Acad. Sci. U.S.A.*, 72: 2274-2278, 1975). Four organisms of this kind have now been characterized. The gene (designated *pss*) which is altered in these mutants is closely linked to the *nadB* locus near minute 49 on the *E. coli* chromosome.

Strains carrying the *pss-8* mutation do not grow at elevated temperatures and have low levels of an altered synthetase in cell extracts. An analysis of several hundred transductants and temperature-resistant revertants reveals that the *pss-8* mutation is responsible both for the enzyme defect and for the phenotype. When a *pss-8* mutant is shifted to the nonpermissive temperature, the cells stop dividing and form long filaments. After 3 hours at 44° the level of phosphatidylethanolamine drops from 66% to 32% (percentage of the total lipid phosphorus), while the combined levels of phosphatidylglycerol and cardiolipin rise from 34% to 68%. The antibiotic sensitivity of these mutants is significantly altered.

PHS-6040
(12-75)

Project Description:

Objectives:

The objectives of this project are (1) isolation of mutants in specific enzymes of phospholipid synthesis; (2) mapping the genes altered in these mutants; and (3) characterization of membrane function in such mutant bacteria.

Methods Employed:

Standard bacterial genetics, together with phospholipid and phospholipid-enzyme analyses.

Major Findings:

Phosphatidylserine Synthetase Mutants of Escherichia coli. Mutants of *E. coli* defective in CDP-diglyceride : L-serine phosphatidyltransferase (phosphatidylserine synthetase) can be isolated by a rapid autoradiographic screening assay described previously (Raetz, C. R. H., Proc. Nat. Acad. Sci. U.S.A., 72: 2274-2278, 1975). Four organisms of this kind have now been characterized. The gene (designated pss) which is altered in these mutants is closely linked to the nadB locus near minute 49 on the *E. coli* chromosome.

Strains carrying the pss-8 mutation do not grow at elevated temperatures and have low levels of an altered synthetase in cell extracts. An analysis of several hundred transductants and temperature-resistant revertants reveals that the pss-8 mutation is responsible both for the enzyme defect and for the phenotype. When a pss-8 mutant is shifted to the nonpermissive temperature, the cells stop dividing and form long filaments. After 3 hours at 44° the level of phosphatidylethanolamine drops from 66% to 32% (percentage of the total lipid phosphorus), while the combined levels of phosphatidylglycerol and cardiolipin rise from 34% to 68%. The antibiotic sensitivity of these mutants is significantly altered.

Phosphatidylglycerophosphate Synthetase Mutants. Approximately 20 mutants of this kind have been isolated. Most of them map in the vicinity of the his gene near minute 37. Analysis of phospholipid composition and growth properties is currently in progress.

Significance to Biomedical Research and the Program of the Institute:

The finding that phosphatidylserine synthetase is indispensable to the growth of *E. coli* implies that inhibitors of this enzyme might be novel antibiotics.

Publications:

Raetz, C. R. H.: Isolation of *Escherichia coli* mutants defective in enzymes of membrane lipid synthesis. Proc. Nat. Acad. Sci. U.S.A. 72: 2274-2278, 1975.

Raetz, C. R. H.: Phosphatidylserine synthetase mutants of *Escherichia coli*: genetic mapping and membrane phospholipid composition. J. Biol. Chem., in press.

Wickner, S. H., Wickner, R. B., and Raetz, C. R. H.: Overproduction of dna gene products by *Escherichia coli* strains carrying hybrid ColE1 plasmids. Biochem. Biophys. Res. Commun., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,710-26 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Estimation, Metabolism, and Function of Amines

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

PI:	Herbert Tabor, M.D.	Medical Director (USPHS)	LBP NIAMDD
	and Chief, Laboratory of Biochemical Pharmacology		LBP NIAMDD
	Celia White Tabor, M.D.	Medical Director (USPHS)	LBP NIAMDD
OTHER:	Edmund W. Hafner, Ph.D.	Staff Fellow	LBP NIAMDD
	Murray S. Cohn, Ph.D.	Staff Fellow	LBP NIAMDD

COOPERATING UNITS (if any)

None

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NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.3	3.8	1.5

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

We have continued our investigation of the function of polyamines *in vivo*. We have studied the genetics of polyamine biosynthesis in *E. coli*. Mutants defective in the biosynthesis of adenosylmethionine have been characterized as *metK*$^-$ mutants. A new mutant deficient in the adenosylmethionine decarboxylase has been found. Adenosylmethionine synthetase has been purified from *E. coli*. For these studies we have developed assays useful for the mass screening of cells, both microbial and mammalian, for pathways defective in CO_2 production.

We are studying the unknown cofactor of adenosylmethionine decarboxylase from yeast.

We have developed methods for the automated analysis of glutathione.

PHS-6040
(10-75)

Project Description:

We have developed a method for the rapid screening of large numbers of cells for mutants defective in any reaction which produces CO_2. This method depends on trapping $^{14}CO_2$ from a ^{14}C-labeled substrate on $Ba(OH)_2$-impregnated paper over a 96-well Falcon plate. With this technique we have isolated a mutant of *E. coli* that has only 5-10% of the normal adenosylmethionine decarboxylase activity, and only about 10% of the wild-type spermidine content. We are studying the effects of this deficiency in *E. coli*. We have also used this technique to screen for transductants, and thus we have mapped the position of this decarboxylase (about 2 1/2 minutes).

We have continued our studies on the adenosylmethionine synthetase of *E. coli*. This enzyme has wide significance in the total biochemistry of *E. coli*, since the adenosylmethionine not only serves as a precursor for polyamines, but serves as the methyl donor in methylation reactions. We have purified this enzyme to homogeneity and are studying the reaction mechanism. We have also isolated mutants in the synthesis of this enzyme, mapped the position by standard genetic techniques, and studied the effects on growth and polyamine metabolism. We have obtained a temperature-sensitive enzyme in a mutant of *E. coli*, which suggests that the *metK* gene is the structural gene for this enzyme, and have carried out mapping experiments with this mutant.

We have previously shown that *E. coli* adenosylmethionine decarboxylase belongs to a class of enzymes recently described that contains novel carboxyl cofactors. We demonstrated that this prokaryotic enzyme contains pyruvate. We have now purified the yeast adenosylmethionine decarboxylase to characterize its carbonyl-containing cofactor. Preliminary studies indicate that opposed to published data for the eurkaryotic enzyme, this enzyme has no pyridoxal phosphate.

We have continued to develop methods for the automated analysis of acidic ninhydrin-positive molecules with an anion-exchange resin. We have separated glutathione, and various derivatives of glutathione, from other closely related thiol groups in order to extend our studies on the biosynthesis of glutathionylspermidine.

We are also using a similar method for the analysis of γ-carboxyglutamate, a newly-described glutamic acid derivative in prothrombin.

Publications:

Tabor, C. W., and Tabor, H.: 1,4-Diaminobutane (putrescine), spermidine, and spermine. Annu. Rev. Biochem. 45, in press.

Tabor, H., and Tabor, C. W.: Glutathionylspermidine in *Escherichia coli*. Ital. J. Biochem. 25: 70-76, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 24,940-03 LBP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mechanisms of Inheritance in *Saccharomyces cerevisiae*

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

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

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

Strains of *Saccharomyces cerevisiae* (yeast) carrying a small double-stranded RNA species (the killer plasmid) secrete a glycoprotein toxin which is lethal only to strains not carrying this plasmid.

We have located 11 chromosomal genes needed to maintain or replicate the killer plasmid.

We have observed bypass of the need for these chromosomal genes by other changes (called KRB for killer replication bypass). In some instances these changes are dominant single-site changes at chromosome centromeres which are unstable to heat. Other KRB changes are unstable to mating and result in changes of the ds RNA species present in the cell.

PHS-6040
(12-75)

Project Description:

Strains of the eukaryote *Saccharomyces cerevisiae* (yeast) carrying a small double-stranded RNA species (the killer plasmid) in virus-like particles secrete a glycoprotein toxin which is lethal only to strains not carrying this plasmid.

Chromosomal Genes Needed for Plasmid Maintenance or Replication. We have isolated mutants in nine chromosomal genes essential for replication or maintenance of the killer plasmid, called mak1 through mak9. Eight of these genes have been mapped. Mak4 and mak5 are on chromosome II; mak1 and mak8 are on chromosome XV; mak3 and mak6 are on chromosome XVI; mak7 is on chromosome VIII; and mak9 is on chromosome XI. We have not yet located mak2. Two other chromosomal genes, m and pets, have been previously shown to be required for replication or maintenance of the killer plasmid.

One allele of mak1 results in temperature sensitivity for host growth. We find that two independent pets isolates also result in the petite phenotype (due to loss of mitochondrial DNA) as well as temperature sensitivity for growth. Pets is not essential, however, for maintenance of the ψ plasmid, a nonchromosomal genome which increases the level of ochre suppression in strains carrying it.

Wild-type killer strains have been reported to carry two species of double-stranded RNA of 2.5×10^6 and 1.4×10^6 molecular weight (designated L and M, respectively); wild-type nonkillers carried only L. We estimate the size of the L and M species at 3.0×10^6 and 1.7×10^6 daltons, respectively. We have also detected a third species of double-stranded RNA of molecular weight 3.8×10^6 (XL) present in all killer and nonkiller strains examined.

Mutation of any of mak1 through mak8 results in loss of the killer-associated species of double-stranded RNA (M; 1.7×10^6 daltons). These mutants retain both the L species (3.0×10^6 daltons) and the XL species (3.8×10^6 daltons) of double-stranded RNA, and have acquired two new minor RNA species.

We have detected dominant chromosomal changes (called KRB for killer replication bypass and located, in one case, near the centromere of chromosome X) which bypass three different mutations in distant plasmid replication genes (pets-1, pets-2, mak7-1). Mak7-1 KRB strains are isolated as stable K^+R^+ sectors of predominantly K^-R^- segregants from the cross of mak7-1 with a wild-type killer. These occur with surprisingly great frequency. Strains mutant in pets have three phenotypes: (a) loss of the killer plasmid resulting in inability to kill or resist being killed; (b) loss of mitochondrial DNA; and (c) temperature sensitivity for growth. KRB bypasses only the inability to maintain the killer plasmid. Heat or cycloheximide readily induces conversion of mak7-1 KRB strains from killers to nonkillers with concomitant disappearance of KRB as judged by further crosses. Known amber and ochre suppressors do not suppress pets-1, pets-2, or mak7-1. KRB could conceivably be an integrated DNA copy of the killer ds RNA plasmid or a derepressed alternate plasmid replication apparatus.

Killer strains defective in other plasmid replication genes (m, pets, mak4, mak5, or mak6) have been isolated as above and are mitotically stable.

The m⁻ killers are especially intriguing in that mating these strains reverses their killer replication bypass change. One of these strains has an approximate 20-fold increase in its content of the 1.7 x 10^6 dalton ds RNA (M).

Significance to Biomedical Research and the Program of the Institute:

The killer plasmid appears to be composed of two species of double-stranded RNA and is associated with virus-like particles. While double-stranded RNA viruses are ubiquitous, infecting bacteria, fungi, plants, insects, and mammals (including at least two pathogens infecting man), this is the first example of a plasmid with a double-stranded RNA genome. The distinction may, however, be purely formal. This system provides a simple means of investigating host-virus genetic relationships as well as the mechanisms of replication, segregation, recombination, and expression of such plasmids.

Mutations of the mak type are analogous to host resistance mutations, i.e., host mutations which leave the virus unable to replicate because the host is not supplying components needed for this process. The KRB changes may represent means which the virus (plasmid) can seize to circumvent these host blocks to its replication (or maintenance). These may prove to be analogous to viral integration or "persistent infection" in higher eukaryotes.

Publications:

Leibowitz, M. J., and Wickner, R. B.: A chromosomal gene required for killer plasmid expression, mating, and sporulation in *Saccharomyces cerevisiae*. Proc. Nat. Acad. Sci. U.S.A., in press.

Wickner, R. B.: Mutants of *Saccharomyces cerevisiae* that incorporate deoxythymidine 5'-monophosphate into DNA *in vivo*. In Prescott, D. M. (Ed.): Methods in Cell Biology. New York, Academic Press, 1975, Vol. 11, Yeast Cells, pp. 295-302.

Wickner, R. B.: Mutants of the killer plasmid of *Saccharomyces cerevisiae* dependent on chromosomal diploidy for expression and maintenance. Genetics 82: 273-285, 1976.

Wickner, R. B.: The killer of *Saccharomyces cerevisiae*: a double-stranded RNA plasmid. Bacteriol. Rev., in press.

Wickner, R. B., and Leibowitz, M. J.: Chromosomal genes essential for replication of a double-stranded RNA plasmid of *Saccharomyces cerevisiae*: the killer character of yeast. J. Mol. Biol., in press.

Wickner, R. B., and Leibowitz, M. J.: Two chromosomal genes required for killing expression in killer strains of *Saccharomyces cerevisiae*. Genetics 82: 429-442, 1976.

Wickner, S. H., Wickner, R. B., and Raetz, C. R. H.: Overproduction of dna gene products by *Escherichia coli* strains carrying hybrid ColE1 plasmids. Biochem. Biophys. Res. Commun. 70: 389-396, 1976.

ANNUAL REPORT OF THE LABORATORY OF CHEMICAL PHYSICS,
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

The detailed molecular architecture of macromolecules of biological importance is being studied in this Laboratory by correlating quantum theoretical calculations with spectroscopic techniques. At the moment most of the work revolves around polynucleic acids and around porphyrin systems, hemoglobin, cytochrome P450 and related model compounds.

Eaton and Hanson, for example, using extended Hückel theory, have calculated the molecular orbitals for an imidazole-iron-porphyrin oxygen complex with results from which it is concluded that pi bonding (mixed iron and oxygen orbitals) makes an important contribution to the stability of oxyghemoglobin. In the case of model compounds of cytochrome P450, there are striking differences calculated between molecules liganded to mercaptides and mercaptans, and these results are consistent with spectral data, leading to the expectation that new insights will be provided into how these molecules perform their biological function. Meanwhile, Kon, Sato and Rispin have been involved with models of cytochrome P450 which do not involve any sulfur ligand. A complex theoretical treatment is demonstrating that the electron paramagnetic resonance of a model Fe(III) compound related to cytochrome P450 which, last year, was shown experimentally to arise from severe rhombic distortion of the model compound in the crystal, is theoretically explicable. Additional work on free radicals in P450 system demonstrates that the enzyme mediated reaction does produce a radical but the source of the radical is atmospheric oxygen.

Charney and Chen extended studies of the solution behavior of DNA and polynucleotides to polycytidilic acid. In this case, however, the spectroscopic properties are somewhat different, and they have demonstrated the existence of a transition at long wavelengths over which considerable uncertainty has existed in the literature.

Macromolecular assembly is a relatively new area of study. Last year, Eaton, Hofrichter, and Ross did the first work on kinetics of the polymerization of sickle cell hemoglobin. This year Hofrichter has constructed a laser light scattering photometer which is capable of resolving changes in scattered intensity as small as one part in 10^{10} of the incident light. Initial results with this instrument show that the technique is capable of observing hemoglobin S aggregation at levels at least 100-fold lower than those detectable by the other techniques used to study gelation. As a consequence it should be possible to study the early phases of the aggregation in an attempt to understand the initiation reactions.

Eaton, Hofrichter, and Ross (LMB) have continued their exploration of sickle cell hemoglobin gelation using a variety of physical techniques, including measurements of turbidity, birefringence, heat absorption and water proton magnetic resonance linewidths. Their kinetic and thermodynamic studies have confirmed the supersaturation equation which was used earlier to predict the rate of gelation *in vivo*. From the correlation between clinical severity and *in vitro* gelation experiments, they have proposed that the delay time of

gelation must be lengthened by at least 20-fold to obtain a therapeutic effect. Since they have found that urea, at concentrations that can be achieved in patients, lengthens the delay time by only a factor of two, Eaton and colleagues have suggested that the maintenance oral urea therapy would most probably not be an effective drug for the treatment of sickle cell disease.

Underlying the spectral properties of these larger molecular systems are the structure and spectra of the small moieties of which they consist. Two such studies have been made. In one, McDiarmid has studied the electronic transitions of UF_6 from 1200-4200 Å, observing 8 electronic transitions in this region which can be correlated with those of UF_6^-, UCl_6^-, MoF_6 and WF_6. Studies on isobutene are also underway. In the other, as the result of newly available experimental data, Charney and Rosenfield have examined theoretically the chiral effects of substituents allylic to the end carbons of diene systems and have found that such major differences exist between hydrogen and carbon substituents that the results may give new insights into the quantum mechanical methods themselves.

Raman spectroscopy offers a potentially valuable approach to examining dynamical properties of natural and artificial membrane bilayer assemblies since no membrane perturbing agent is introduced by the technique. This is particularly crucial in investigating conformation changes induced in bilayer systems. Levin and his co-workers have determined Raman spectroscopic frequency differences between selected carbon-carbon stretching modes of lipid hydrocarbon chains as a function of temperature for use in monitoring lipid phase transition behavior and acyl chain disorder in both multilamellar and single-wall vesicles. Transition temperatures detected by this procedure for pure dipalmitoyl and dimyristoyl phosphatidylcholine multilayers were observed at 39±1°C and 23±1°C, respectively. Although the phase transition for unilamellar vesicles of dipalmitoyl phosphatidylcholine occurred at nearly the same temperature as the multilayers, the crystal-liquid crystalline transition for the single-shell vesicles appeared to span a slightly broader temperature range, a characteristic consistent with irregularities in the packing arrangement of the hydrocarbon chains. Within the precision of the Raman spectroscopic method, the temperature behavior of both the multilamellar and unilamellar dimyristoyl phosphatidylcholine assemblies appeared nearly identical. The temperature profile for the Raman frequency differences of an excess water sonicate of 25 mol percent cholesterol in dipalmitoyl phosphatidylcholine served as an example of the effect upon lipid phase transition characteristics of a bilayer component intercalated between the acyl chains.

Shean has continued studies of experimental models illustrating osmotic water transport across liquid oil membranes, which have been shown to be fairly semi-permeable (i.e. permeable to water and poorly permeable to KCl). Although much smaller than theoretically possible because the magnitude was limited by the model, hydrostatic heads of over 20 cm were obtained.

Studies of the structure of nucleotides have received particular attention. Last year we reported on a proton NMR investigation of association and unexpectedly slow exchange in guanosine-5'-phosphate (GMP). Fisk, Becker and

Miles (LMB) have now examined ^{13}C NMR spectra of the associated form(s) at high magnetic field, where chemically similar but structurally non-equivalent carbon atoms can be differentiated. Qualitatively the ^{13}C spectra confirm the earlier proton NMR data that pointed to a probable structure consisting of 3 or 4 stacked tetrameric GMP units. The exact origin of the observed chemical shift differences is unclear; "ring currents" undoubtedly play a role, but their calculations indicate that ring current effects are inadequate to account for the large shifts observed.

In many cases energy minimization calculations can provide valuable insight into nucleotide conformations, but the validity of the calculation is critically dependent on the extent to which the potential function that is employed reflects the forces that occur in the molecule. Govil has found that in a nucleotide chain the usual threefold symmetric potential function that has been used does not account for oxygen lone-pair repulsions in the 3'-5' O-P-O linkage. Introduction of an additional term of twofold symmetry has been shown to provide a simple and satisfactory way to introduce these effects into the calculation.

In the course of studies establishing the absolute stereochemistry of aromatic metabolites the severe limitations associated with working with small quantities of material available from enzymatic reactions became apparent. In order to overcome these limiations Ziffer and Kabuto undertook a program on the asymmetric synthesis of a number of hydroaromatic alcohols. In one approach they examined the chiral synthesis of some cis and trans diols employing a lithium aluminum:ℓ-darvon (1:2) complex to reduce an α-acetoxy ketone. In a second approach they examined the use of actively fermenting yeast to stereospecifically reduce hydroaromatic ketones to optically active alcohols. The quantities of material and optical purity obtained via this reduction suggest that this method can be used to prepare material for chemical transformation that was not previously available from enzymatic studies.

Weiss and his collaborators have isolated the antibiotics chrysomycin A, $C_{30}H_{30}O_{10}$, and chrysomycin B, $C_{29}H_{30}O_{10}$, as the crystalline, homogeneous diacetates from the previously known crude crystallizate. NMR studies indicate that their structures are identical, except for the replacement of a vinyl group in A, bound to an aromatic ring by a methyl group in B. They have also continued their study of the reaction of dimethyl β-ketoglutarate with 1,2-dicarbonyl compounds to yield products which support previously postulated mechanisms for this reaction. One of the compounds from the reaction of dimethyl β-ketoglutarate with glyoxal has been transformed to a substance whose structure bears analogies to that of the steroids. The readily available compound may be useful as an intermediate in total synthesis of steroids. With cyclohexane-1,3-diones, dimethyl β-ketoglutarate yields partially hydrogenated coumarins, readily transformed into tetrahydroquinolones whose structures suggest possibilities for the synthesis of antimalarial quinolines.

Hagins, Yoshikami (NEI) and Robinson have continued their studies on the cellular mechanism of transduction in vertebrate retinal rods and cones. A new technique for introducing membrane impermeable substances into cells has been used to place hydrogen ion and metal ion buffers into retinal rods. It

has thereby been shown that calcium ions are the probable intracellular transmitter of visual excitation. Purified rod outer segments have been shown to have an ATPase that could provide the energy needed for amplification in visual excitation.

Sharpless and Adams have synthesized and partly characterized several model compounds of retinaldehyde with spectral properties resembling that of vertebrate visual pigment chromophores.

In studies on basic mechanisms underlying the occurrence of hyperbilirubinemia in man and animals at high altitude (continuous or intermittent hypoxic exposures) Altland and his co-workers have found that the main factors are increased destruction of erythrocytes associated with severe polycythemia (hematocrit above 68%), increased intravascular hemolysis due to increased blood viscosity rather than altered erythrocyte fragility and the apparent inability of the liver to handle increased levels of bilirubin.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29000-04-LCP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Nuclear magnetic resonance: New methods and molecular structure determination

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

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LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7	6	1

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

NMR studies of nucleotide conformation and nucleotide interactions concentrated on the elucidation of structure of the associated form of guanosine-5'-phosphate at neutral pH. Related theoretical studies showed that account must be taken of lone-pair electron repulsions in the 3'-5' O-P-O linkage of polynucleotides.

A new multi-nuclear NMR spectrometer was completed and placed in operation, and significant technical improvements were introduced, especially in the study of dilute aqueous solutions.

Project No.: Z01-AM-29000-04-LCP

Project Description:

Objectives:

1. Elucidation 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.

Major Findings and Future Plans:

Studies of the structure of nucleotides have received particular attention. Last year we reported on a proton NMR investigation of association and unexpectedly slow exchange in guanosine-5'-phosphate (GMP). We have now examined ^{13}C NMR spectra of the associated form(s) at high magnetic field, where chemically similar but structurally non-equivalent carbon atoms can be differentiated. Qualitatively the ^{13}C spectra confirm the earlier proton NMR data that pointed to a probable structure consisting of 3 or 4 stacked tetrameric GMP units. The exact origin of the observed chemical shift differences is unclear; "ring currents" undoubtedly play a role, but our calculations indicate that ring current effects are inadequate to account for the large shifts observed. ^{13}C NMR relaxation time studies are planned in an effort to firmly establish the structure. (Fisk, Miles, Becker).

In many cases energy minimization calculations can provide valuable insight into nucleotide conformations, but the validity of the calculation is critically dependent on the extent to which the potential function that is employed reflects the forces that occur in the molecule. We have found that in a nucleotide chain the usual threefold symmetric potential function that has been used does not account for oxygen lone-pair repulsions in the 3'-5' O-P-O linkage. Our introduction of an additional term of twofold symmetry has been shown to provide a simple and satisfactory way to introduce these effects into the calculation. During the next year other refined potential functions will be applied to the elucidation of conformation in interacting nucleotides and in phospholipids, for which a similar theoretical approach is appropriate. ^{1}H and ^{13}C NMR spectra will also provide key parameters in defining the detailed molecular structures. (Govil).

In most bioreactions, steady-state conditions are not achieved and thus kinetics rather than thermodynamics plays the predominant role. Local variations in concentration, polarity surrounding and conformation at a binding site, activation of complexed, free and bound substrate, etc., provide a cascaded series of kinetic steps which highly define products. Similar but very limited kinetic sorting of nuclear spins occurs for in vitro radical pair reactions in magnetic fields. However the magnetic polarization generated disappears in a few seconds via nuclear relaxation. Thus rapid measurement of high resolution magnetic resonance on in vivo biological reactions may provide a means of detecting specific chemical transformations and has the special attribute of indicating radical pair pathways which may relate to abnormal bioprocesses.

A steady state approach to evaluating such measurement potential is being attempted by using a continuous flow manifold on high resolution C-13 spectrometers. Because of the potential extension to small peptides, amide detection thru nuclear polarization when acting as a solvated electron transfer agent on phenol photolysis in aqueous alkali is being investigated using the C-13 FT spectrometer at NRL in collaboration with Dr. W. Moniz. Argon ebulation has minimized oxygen radical quenching. Disasterous overflow problems and suction aeration were eliminated by careful control of input and output flows for both recycled and single pass configurations with a multichannel pump. Esr evidence for significant radical concentrations has been observed in collaboration with Dr. H. Kon, but C-13 polarization of the system is still uncertain in the chosen pH 11 aqueous 0.1 M phenol solutions. Efforts will continue on this system and on reagent mixing induced reactions for which a flow manifold is under construction to use on the TT-14 C-13 FT spectrometer in this laboratory. (Lepley).

Further development of our high field NMR center continued with a number of improvements in instrumentation and techniques. A new multinuclear spectrometer was completed and put into operation--initially for ^{13}C NMR but ultimately to be used for study of a wide range of nuclei. In addition, a number of instrumental modifications and changes in computer programs were carried out to appreciably enhance our capabilities in ^{1}H NMR at high field. One area that received particular attention was the development and evaluation of several methods for minimizing interference from the solvent signal while carrying out NMR studies of samples in dilute aqueous solution. (Tschudin, Clem, Fisk, Becker).

Publications:

Pinnavaia, T. J., Miles, H. T. and Becker, E. D.: Self-assembled 5'-guanosine monophosphate. Nuclear magnetic resonance evidence for a regular, ordered structure and slow chemical exchange. J. Amer. Chem. Soc. 97: 7198-7200, 1975.

Govil, G.: Conformational structure of polynucleotides around the O-P bonds: Refined parameters for CPF calculations. Biopolymers. In press.

Eaton, W. A., Hofrichter, J., Ross, P. D., Tschudin, R. G. and Becker, E. D.: Comparison of sickle cell hemoglobin gelation kinetics measured by NMR and optical methods. Biochem. Biophys. Res. Comm. 69: 538-547, 1976.

REPORTED IN PRESS - 1975

Tucker, E. E., Clem, T. R., Seeman, J. I. and Becker, E. D.: Carbon-13 nuclear magnetic resonance relaxation in hydrogen bonded tert-butyl alcohol and phenol. J. Phys. Chem. 79: 1005-1008, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29001-04-LCP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Molecular Dynamics

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

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0

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

Raman spectroscopic frequency differences between selected carbon-carbon stretching modes of lipid hydrocarbon chains were determined as a function of temperature for use in monitoring lipid phase transition behavior and acyl chain disorder in both multilamellar and single-wall vesicles. Transition temperatures detected by this procedure for pure dipalmitoyl and dimyristoyl phosphatidylcholine multilayers were observed at 39±1°C and 23±1°C, respectively. Although the phase transition for unilamellar vesicles of dipalmitoyl phosphatidylcholine occurred at nearly the same temperature as the multilayers, the crystal-liquid crystalline transition for the single-shell vesicles appeared to span a slightly broader temperature range, a characteristic consistent with irregularities in the packing arrangement of the hydrocarbon chains. Within the precision of the Raman spectroscopic method, the temperature behavior of both the multilamellar and unilamellar dimyristoyl phosphatidylcholine assemblies appeared nearly identical. The temperature profile for the Raman frequency differences of an excess water sonicate of 25 mol percent cholesterol in dipalmitoyl phosphatidylcholine served as an example of the effect upon lipid phase transition characteristics of a bilayer component intercalated between the acyl chains.

Project No.: Z01-AM-29001-04-LCP

Project Description:

Within the project area of molecular dynamics our objectives are to probe, clarify and interpret the vibrational characteristics of selected moderate to large molecular systems through the following approaches: (a) Analyses of Raman and infrared spectra, (b) determination of reliable intramolecular force fields, and (c) interpretation of molecular charge distribution (infrared and Raman intensity) parameters.

A variety of laser Raman and infrared spectroscopic procedures provide the required experimental data for given vibrational analyses. The intramolecular force field and vibrational intensity computations are necessarily performed on a large digital computer.

We shall divide the results of the past year's studies into two subsections: (a) Intramolecular force fields and exchange processes for MX_5 molecules, and (b) analysis of the vibrational Raman and infrared spectra of model biological membrane assemblies.

(a) Intramolecular force field determinations

In assessing the energetics and pathways of intramolecular exchange processes for stereochemically nonrigid MF_5 molecules, it is of interest to develop potential functions based upon the observed vibrational spectra of the low frequency modes which participate in the axial-equatorial fluorine atom rearrangements. We extended our previous study of PF_5 to the interpretation of the gas-phase Raman spectra of the ν_7 vibrational transitions of AsF_5 and VF_5. In contrast to our earlier investigation, we determine a two dimensional, anharmonic potential function from the observed spectra that is now both constrained to conform to the double minimum class and generalized to consider the dimensionless eigenvalues of any MX_5 (D_{3h}) molecule in terms of a single cubic potential constant. The intramolecular exchange barrier heights, determined by the double minimum potentials, lie between 2.84-3.26 kcal/mol for PF_5, 2.16-2.47 kcal/mol for AsF_5 and 1.22-1.54 for VF_5. The dynamics of the fluorine atom interchange pathways suggest that these trigonal bipyramidal (D_{3h}) molecules form C_{4v} intermediates by initially displacing the equatorial fluorine atoms and then by mixing in the axial fluorine distortions as the intramolecular exchange proceeds. (Abramowitz, Bernstein, Levin)

(b) Analysis of the vibrational spectra of model biological membrane assemblies

(1) Raman spectroscopic frequency differences between selected carbon-carbon stretching modes of lipid hydrocarbon chains were determined as a function of temperature for use in monitoring lipid phase transition behavior and acyl chain disorder in both multilamellar and single-wall vesicles. Transition temperatures detected by this procedure for pure dipalmitoyl and dimyristoyl phosphatidylcholine multilayers were observed at 39 ± 1°C and

23±1°C, respectively. Although the phase transition for unilamellar vesicles of dipalmitoyl phosphatidylcholine occurred at nearly the same temperature as the multilayers, the crystal-liquid crystalline transition for the single-shell vesicles appeared to span a slightly broader temperature range, a characteristic consistent with irregularities in the packing arrangement of the hydrocarbon chains. Within the precision of the Raman spectroscopic method, the temperature behavior of both the multilamellar and unilamellar dimyristoyl phosphatidylcholine assemblies appeared nearly identical. The temperature profile for the Raman frequency differences of an excess water sonicate of 25 mol percent cholesterol in dipalmitoyl phosphatidylcholine served as an example of the effect upon lipid phase transition characteristics of a bilayer component intercalated between the acyl chains. For this cholesterol-lipid system the phase transition was broadened over a 30°C temperature range in contrast to the narrow 2-4°C range observed for pure multilayer and single-shell vesicle particles. (Spiker and Levin)

(2) In order to clarify the effect of bilayer curvature upon phospholipid conformation, vibrational Raman spectra were recorded for dipalmitoyl and dimyristoyl phosphatidylcholine in the gel state for both multilayer and single-wall vesicle assemblies. An intensity comparison, based upon use of an internal standard, between the two classes of bilayer systems reflected a decrease in peak height intensity for the hydrocarbon chain vibrational transitions in the single shell vesicle form. No intensity change between bilayer form was detected in the two observed head group modes. Trends in the peak height intensity ratios for the 1100 cm^{-1} C-C stretching vibrations indicated an increase in hydrocarbon chain trans-gauche isomerization for the vesicles in comparison to multilayer arrangements. The sensitivity of the methylene C-H stretching modes to interchain disorder was demonstrated by comparisons of intensity patterns in the 2900 cm^{-1} region to the intensity characteristics of the C-C stretching region for polycrystalline, multilayer and vesicle materials. Examination of various intensity ratios for cholesterol doped dipalmitoyl phosphatidylcholine bilayers indicated that while 25 mol % cholesterol increased the trans-gauche isomerization in multilayers, no comparable effect was observed for the vesicle forms. In contrast, the CH_2 twisting/CH_2 deformation intensity ratios for the cholesterol containing systems suggested that some type of additional interchain perturbation occurs in the vesicle aggregations. (Spiker and Levin)

(3) For determining the infrared and Raman spectra of membrane related systems, a method is developed for incorporating phospholipid bilayer assemblies in a clay matrix to form ultra-thin, self-supporting films. These films, containing stabilized bilayers arranged between the silicate layers of hectorite, are in the shape of discs which measure 2 cm in diameter and 25 microns thick and require approximately 1-2 mg of phospholipid for preparation. Although several spectral regions below 1100 cm^{-1} are masked by the host clay, both head group and acyl chain vibrations may be conveniently observed and monitored for phospholipid conformational changes. (Spiker, Pinnavaia and Levin)

(4) As a prelude to investigating lipid-protein interactions involving various metalloporphyrins chromophores, we examined the resonance Raman spectra of the triclinic and tetragonal crystalline forms of α, β, γ, δ tetraphenylporphinatocobalt (II) [Co(II)TPP]. General interest in the resonance Raman spectra of metalloporphyrins stems from the observation that appropriate transitions in the 1350-1650 cm^{-1} region are sensitive to changes in structure, oxidation state and spin state. Examination of the two crystalline forms revealed distinctly different resonance enhanced spectra in two regions, 950-1050 cm^{-1} and 1500-1600 cm^{-1}. Interestingly, as a consequence of the symmetry of the forms of Co(II)TPP, these systems are the first metalloporphyrins not to exhibit anomolously polarized lines characteristic of planar cores. Several structural conclusions were deduced from the resonance Raman spectra: (a) the tetragonal form exhibits S_4 symmetry [later verified by x-ray crystallography, C. Kabuto and J. Silverton, private communication] and (b) the triclinic form also exhibits spectra consistent with S_4 local symmetry. The differences in the two structures reflect changes in the relative tilting of the phenyl and pyrole rings between the systems. On the basis of contributions from the phenyl group to the resonance Raman spectra of the triclinic crystal, we conclude that the phenyl groups in this form tend more toward planarity than in the tetragonal form. Both crystalline structures give the same solution spectra. The similarity between the solution and triclinic crystalline spectra suggests that the more coplanar structure is the more stable conformation. (Yellin and Levin)

(5) Raman spectra of dimyristoyl, dipalmitoyl, and distearoyl phosphatidylcholine bilayers, with acyl chain lengths of 14, 16 and 18 carbon atoms, respectively, were examined as a function of temperature in the gel state from about -180°C to near the phase transition point. An analysis of the 1100 cm^{-1} C-C stretching region over this temperature range allows a determination of the enthalpy differences (ΔH) between the all-trans hydrocarbon conformation and the gauche isomers that are formed as the phase transition is approached. Enthalpy differences between the all-trans and disordered systems for the dimyristoyl, dipalmitoyl and distearoyl molecules are 3.1 ± 0.5, 3.4 ± 0.5 and 10.4 ± 1.0 kcal/mol, respectively. These quantities may be associated with a value of about 1-1.5 kcal/mole per gauche structure. (Yellin and Levin)

(6) The polyene antibiotic amphotericin B is thought to form channels in lipid membranes containing sterols. Resonance enhanced and normal vibrational Raman spectra for both the antibiotic and membrane components were observed for a dimyristoyl lecithin (DML)-cholesterol-amphotericin B system. The lipid-sterol environment lowers the polyene antibiotic C=C stretching frequency, compared to that of the solid or solution, and significantly increases the intensity of the 1001 cm^{-1} C-C-H bending mode. Since this C-C-H deformation is coupled to C-C and C=C skeletal stretching modes, the resonance enhancement suggests that the polyene chain changes conformation from a slightly twisted structure to a more planar form. The stoichiometry of binding of cholesterol to amphotericin B was calculated as 1:1 at 25°C

by monitoring the DML phase transition in the 2900 cm^{-1} CH_2 stretching region. Differential scanning calorimetry confirmed this conclusion.

Addition of amphotericin B to pure DML vesicles increases the intensity of the 2880 cm^{-1}/2850 cm^{-1} (asym. methylene stretch/sym. methylene stretch) ratio which implies a stiffening or ordering of the lipid acyl chains perhaps by surface binding of the antibiotic. For DML-cholesterol multilayers, addition of amphotericin B raises the intensity of the 2935 cm^{-1} terminal methyl stretching mode. This effect confirms penetration to the interior of the bilayer by amphotericin B. The intensity of the 2935 cm^{-1} mode increases at lower temperature, which is in accord with the temperature dependence of amphotericin B channels. (Bunow and Levin)

Significance to Biomedical Research and the Program of the Institute:

Raman spectroscopy offers a potentially valuable approach to examining dynamical properties of natural and artificial membrane bilayer assemblies since no membrane perturbing agent is introducted by the technique. This is particularly crucial in investigating conformation changes induced in bilayer systems.

Since experimentally determined vibrational frequencies and charge distribution parameters reflect the sum of several subtle electronic and nuclear contributions, force field determinations and absolute intensity studies permit both quantitative and qualitative descriptions of the dominant terms and create a link between empirical spectroscopic information and bond properties.

Proposed Course of Project:

(1) Interactive normal coordinate computer programs will be used to monitor conformational behavior in selected macromolecules.

(2) Investigations of the vibrational behavior of both vesicle and multilamellar bilayer systems will continue with emphasis on (a) lipid-protein interactions by means of resonance Raman techniques using tunable dye laser procedures, (b) compositional bilayer asymmetries induced by the effects of surface curvature, and (c) the use of appropriately deuterated lipid species for clarifying vibrational assignments of both head group and acyl chain modes.

Publications:

Bernstein, L. S., Abramowitz, S. and Levin, I. W.: Potential function axial-equatorial fluorine atom exchange in PF_5, AsF_5 and VF_5. J. Chem. Phys. 64: 3228-3236, 1975.

Pearce, R. A. R. and Levin, I. W.: Role of redundant coordinates in evaluating skeletal force constants in simple cyclic systems. Spectrochimica Acta. In press.

Spiker, R. C., Jr. and Levin, I. W.: Phase transitions of phospholipid single-wall vesicles and multilayers: measurement by vibrational Raman spectroscopic frequency differences. Biochim. Biophys. Acta. In press.

REPORTED IN PRESS 1975

Levin, I. W. and Pearce, R. A. R.: Intramolecular force field calculations methods and applications. In Durig, J. R.: Vibrational Spectra and Structure, Vol. 4. Elsevier Scientific Publishing Co., 1975, pp. 101-186.

Bernstein, L. S., Kim, J. J., Pitzer, K. S., Abramowitz, S. and Levin, I. W.: Potential function for the ν_7 vibration of phosphous pentafluoride. J. Chem. Phys. 62: 3671-3675, 1975.

Spiker, R. C., Jr. and Levin, I. W.: Raman spectra and vibrational assignments for dipalmitoyl phosphatidylcholine and structurally related molecules. Biochim. Biophys. Acta 388: 361-373, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29002-03-LCP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Chemistry and biosynthesis of natural compounds, and instrumental methods used in their study

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

PI:	Ulrich Weiss	Research Chemist	A LCP
OTHER:	Kunitoshi Yoshihira	Visiting Scientist	A LCP
	Surendra P. Bhatnagar	Visiting Associate	A LCP

COOPERATING UNITS (if any)

James M. Cook, Department of Chemistry, University of Wisconsin, Milwaukee

LAB/BRANCH
Laboratory of Chemical Physics
SECTION
Section on Molecular Biophysics
INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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

The antibiotics chrysomycin A, $C_{30}H_{30}O_{10}$, and chrysomycin B, $C_{29}H_{30}O_{10}$, have been isolated as the crystalline, homogeneous diacetates from the previously known crude crystallizate. NMR studies indicate that their structures are identical, except for the replacement of a vinyl group in A, bound to an aromatic ring, by a methyl group in B. The detailed chemical structure of these antibiotics is under study.

Work on the synthetic preparation of halogen-derivatives of analgesics of the codeine series has been initiated.

Continued study of the reaction of dimethyl β-ketoglutarate with 1,2-dicarbonyl compounds has yielded products which support previously postulated mechanisms for this reaction (work in cooperation with Dr. James M. Cook). One of the compounds from the reaction of dimethyl β-ketoglutarate with glyoxal has been transformed to a substance whose structure bears analogies to that of the steroids. The readily available compound may be useful as an intermediate in total synthesis of steroids.

With cyclohexane-1,3-diones, dimethyl β-ketoglutarate yields partially hydrogenated coumarins, readily transformed into tetrahydroquinolones whose structures suggest possibilities for the synthesis of antimalarial quinolines (work in cooperation with Dr. Cook).

Project No.: Z01-AM-29002-03-LCP

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. Synthesis of compounds of medical or biological interest.

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 and Future Plans:

1. Fungal Metabolites

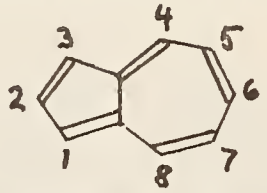
a) Chrysomycins A and B (Dr. Yoshihira). Acetylation of the crude crystallizate of chrysomycin, followed by careful chromatography, has permitted the isolation of the pure, crystalline diacetates of chrysomycins A and B, the latter being a minor component of the mixture (see report for 1974/75).

The chemical nature of these antibiotics has been explored with the help of modern instrumental techniques. Chrysomycins A and B were found to have the empirical formulae $C_{30}H_{30}O_{10}$ and $C_{29}H_{30}O_{10}$, respectively. Detailed study of the 220 mHz NMR spectra has shown that the structures of the two antibiotics are identical except for the replacement of a vinyl group, $-CH=CH_2$, of compound A by a methyl in compound B; those groups are attached to a mono-methoxylated aromatic ring. Replacement of vinyl by methyl is quite unusual; it is interesting from the viewpoint of biosynthesis.

Several other groups have been identified by NMR: double bonds, one keto group, methyls, ethyls, hydroxyls, ether oxygen, etc. However, despite much effort, it has not been possible to assemble those units unequivocally into a unique structure. Several derivatives (diacetates, mono- and bis-bromophenylurethanes) have been obtained in crystalline form, but these crystals were invariably too small to be suitable for x-ray crystallographic study.

Attempts to find a derivative capable of forming crystals of a size suitable for x-ray work are being continued by Dr. Yoshihira at the National Institute of Hygienic Sciences, Tokyo, Japan.

b) Azulene Pigments from Basidiomycetes of the Genus Lactarius (Dr. Yoshihira). Pigments containing the bicyclic ring system of azulene



have been isolated repeatedly from plants, especially from their essential oils; they are often responsible for the therapeutic properties of these oils. However, most of the azulenes isolated so far are artefacts, formed during distillation of the oils from precursors with the carbon skeleton of azulene but richer in hydrogen and usually oxygenated.

We had observed earlier that the sky-blue milky juice of young specimens of a mushroom, Lactarius indigo, is due to a preformed azulene (see report for 1969/70). The excessive instability of this pigment, and the difficulty of finding such young specimens, prevented closer study at that time.

We have now had occasion to examine a few older specimens of L. indigo (no longer yielding any milky juice), and two hitherto uninvestigated species with wine-red juice; they were provided by Dr. K. McKnight, U.S. Department of Agriculture, Beltsville, Md., with the request that we examine their pigments as a possible help in his taxonomic studies of this large and complex genus; the rarity of authentic natural azulenes, and their biological action, made it seem worthwhile to undertake a limited study.

The mature specimens of L. indigo yielded the purple lactaroviolin (4-methyl-7-isopropenylazulene-1-aldehyde), first isolated in 1935 from the edible L. deliciosus, but apparently not present as such in that fungus. In addition, trace amounts of a new pigment were isolated as scarlet crystals. The compound was identified by NMR and mass spectrometry as 4-methyl-7-acetoazulene-1-aldehyde. Unlike the majority of known azulenes from natural sources, the substance is thus not derived from guaiazulene, $C_{15}H_{18}$, (1,4-dimethyl-7-isopropyl azulene), but from chamazulene, $C_{14}H_{16}$ (1,4-dimethyl-7-ethylazulene).

The two species with wine-red juice, L. subpurpureus and L. paradoxus, gave minute amounts of two new, scarlet azulene aldehydes in crystalline form, identified by spectroscopy as 4-methyl-7-isopropylazulene-1-aldehyde (dihydrolactaroviolin) and 4-methyl-7-(α-hydroxyisopropyl)azulene-1-aldehyde, respectively. L. paradoxus also yielded a blue azulene in amounts too small for complete characterization; it seems to be an ether or ester of 1-hydroxymethyl-4-methyl-7-isopropylazulene.

2. Analgesics of the Morphine Series

a) 2-Halogenocodeines and -morphines. 1-Fluorocodeine has been found to resemble codeine itself closely in analgesic action and binding to the narcotics receptor from the central nervous system, while 1-chloro- and 1-bromocodeines have long been known to be significantly weaker analgesics (see reports for 1973/74 and 1974/75). For comparison with these 1-halocodeines, the preparation of 2-fluoro-, chloro-, and bromocodeine is at present being studied by Dr. N. Chatterjie, William Paterson College of New Jersey, under contract with NIAMDD. Information on analgesic action and receptor-binding capacity of these 2-halocodeines should contribute to our understanding of the mechanism of action of drugs of the morphine group.

b) Biomimetic Synthesis of 1-Fluorocodeine (S. P. Bhatnagar). The biosynthesis of morphine from its precursor, the 1-benzyltetrahydroisoquinoline alkaloid reticuline, is now well understood. It proceeds by phenol-oxidation to the morphinadienone salutaridine, which already contains the carbon-nitrogen skeleton of morphine; subsequent reactions produce thebaine, codeine, and morphine. An efficient biomimetic total synthesis (i.e. a laboratory synthesis patterned after the biosynthesis) has long been a goal of organic chemists. While its later steps seem to offer few problems, the phenol-coupling of reticuline or closely related compounds in the laboratory generally yields only little salutaridine; the main product is the aporphine isoboldine, with the morphinadienone isosalutaridine (pallidine) as a relatively major by-product; neither of these compounds can be used for the synthesis of morphine. Tolerable yields (23%) of salutaridine have been obtained so far only when thallium tris-trifluoroacetate was used to bring about the phenol-coupling. This reagent is very undesirable on account of its high cost and dangerous toxicity.

Now formation of both isoboldine and isosalutaridine involves position 6' of reticuline, while this position does not play any role in the formation of salutaridine. It seems possible, therefore, that substituting position 6' with a tightly bound atom or group might steer the phenol-oxidation in direction of salutaridine. As a suitable substituent, the atom of fluorine suggests itself, since it is very strongly held, i.e. not likely to be eliminated, as bromine is in some related reactions. The small size of the fluorine atom may also minimize the danger of steric interference. It is true that there are no methods for removal of fluorine bound to an aromatic ring; however, it so happens that the product of the projected synthesis would be 1-fluorocodeine. The retention of the fluorine throughout the reaction sequence should thus not be objectionable. Work towards the synthesis of 6'-fluororeticuline is under way.

3. Reaction of Dimethyl β-Ketoglutarate with Diketones.

a) Transformations of dimethyl 1,10-dihydroxy-tetracyclo-6.5.0.$0^{2,10}$.$0^{3,7}$]tridecane-5,12-dione 2,8-dicarboxylate* (Dr. K. C. Rice, see report for 1974/75). In spite of its formidable name(s), this compound is very readily accessible, in good yields, by reaction of dimethyl β-ketoglutarate (acetone dicarboxylate) and glyoxal at room temperature in aqueous solution buffered to pH 6.0, and treatment of an intermediate compound with a mixture of sulfuric and acetic acids.

We have found that the mono-acetate of this compound, on being refluxed with excess methanolic sodium methoxide, smoothly gives a new substance. Spectroscopic investigation has shown that during formation of this new compound, one ring has become aromatic, one bond of the complex ring system has been broken, one molecule of acetic acid has been eliminated, and one carbomethoxy group has migrated to a new position in the molecule; the last-named process is uncommon but has good precedent. The ring system of the compound so formed--a substituted hexahydrobenz[e]indene--corresponds to rings B, C, and D of a steroid, with ring B aromatic and substituted in such a way that construction of the missing ring A seems feasible by standard methods. Utilization of the readily available substance as starting material for steroid drugs thus suggests itself. The need for such syntheses is well recognized. For this purpose, our compound offers the advantage of ready preparation from very cheap and abundant commercial starting materials. In addition, its structure suggests possibilities for conversion to a wide variety of different steroid types, and the resolution into optically active material might be convenient. Absence of the methyl group (No. 18) at the juncture of rings C and D, which seems essential for biological activity, is a shortcoming, but methods for its introduction are available; however, they require several steps.

In spite of this complication, our compound seems to offer favorable possibilities for the synthesis of steroids. The Patent Office of HEW is therefore going to seek patent protection for it.

The actual elaboration of the many steps required to convert our compound into a variety of steroid drugs is a problem which would require the efforts of a larger group of research chemists than we have available; consequently, cooperation with some academic institution would be highly desirable. Plans in this direction are being considered.

In continued cooperation with Dr. James M. Cook and his students at the Department of Chemistry, University of Wisconsin, Milwaukee, the

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* It seems that an alternative name: Octahydro-7a,9-dihydroxy-2,6-dioxo-4,8-methanocyclopent[a]indene-3b,8[1H,4H]-dicarboxylate, is in better agreement with the latest rules for naming organic compounds.

reaction of dimethyl β-ketoglutarate with diketones has been investigated further; besides additional 1,2-diketones, some cyclic 1,3-diketones such as 1,3-cyclohexanedione and dimedone [5,5-dimethyl-1,3-cyclohexanedione) have been included in the study.

Most 1,2-diketones give products formed by reaction of one molecule with two molecules of the ester; however, some of them (camphorquinone, benzil, phenanthrenequinone) react with 1:1 stoichiometry. The structures of the resulting compounds support our postulates for the mechanism of formation of the 1:2 adducts.

The two cyclic 1,3-diketones studied so far react in a very different way and in 1:1 ratio, to give oxygen heterocycles (hydrogenated coumarins), which thus become readily accessible. Replacement of the oxygen by nitrogen produces hydrogenated quinolones (carbostyrils). Since it should be possible to convert these compounds into quinolins, a novel synthesis of the latter might result, which would be of value in preparing antimalarials. Detailed study of this possibility is planned.

Publications:

Rice, K. C., Sharpless, N. E., Weiss, U. and Highet, R. J.: The reaction of dimethyl β-ketoglutarate with 1,2-dicarbonyl compounds. III. Exo-tricyclo[5.5.1.$0^{2,6}$.$0^{10,13}$]-tridecane-4,8,12-trione. Tetrahedron Letters: 3763-3766, 1975.

Rice, K. C., Weiss, U., Akiyama, T., Highet, R. J., Lee, T. and Silverton, J. V.: Reaction of dimethyl β-ketoglutarate with 1,2-dicarbonyl compounds. IV. Formation of a complex tetracyclic ring-system in aqueous solution at room temperature. Tetrahedron Letters: 3767-3770, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29003-09-LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The physical chemistry of membranes and complex membrane systems of biological interest

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

PI: Gerald M. Shean Research Chemist A:LCP

COOPERATING UNITS (if any)

Karl Sollner (Scientist Emeritus) A:LCP

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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

Experimental models illustrating osmotic water transport across liquid oil membranes have been studied and shown to be fairly semi-permeable (i.e. permeable to water and poorly permeable to KCl). Although much smaller than theoretically possible because the magnitude was limited by the model, hydrostatic heads of over 20 cm were obtained.

.00275 N rubidium bromide was accumulated across a mosaic liquid ion-exchange membrane consisting of anion permselective and cation permselective parts from a .0001 N RbBr solution. This accumulation which is based on a Donnan equilibrium is 55% of the theoretical maximum for the sytem employed.

Project No: Z01-AM-29003-09-LCP

Project Description:

Objectives:

To prepare and study well-defined synthetic porpous and liquid ion-exchanger membranes and membrane systems with the ultimate aim to provide a rational basis for the understanding of various complex membrane phenomena occurring in living systems.

Methods Employed:

The methods employed were essentially the same as reported previously, including flamephotometry, potentiometric titrations (including the chloridimeter), spectrometry, non-aqueous titrations, etc.

Major Findings:

Progress has continued in assembling and running accumulation models that are capable of extracting and concentrating both cations and anions simultaneously from dilute electrolyte solutions. These models which employ two liquid ion exchanger membranes, one anion permselective and one cation permselective, accumulate ions by exchanging continuously generated "inside" ions for the ions of a different electrolyte solution "outside", e.g. "Outside" Sol'n (Dilute A^+B^- and dilute C^+D^-, their concentrations maintained by flowing thru 200 liters of sol'n) || cation and anion permselective membranes in parallel.|| "Inside" Sol'n (125 ml of more concentrated C^+D^- maintained by adding C^+D^- as needed.)

As a Donnan equilibrium is reached, the ratio of the ions inside and outside should be the same, i.e. A^+B^- is concentrated inside since the ratio of C^+D^- is held constant. Accumulations of RbBr to the extent of 55% of the theoretical expected from a perfect Donnan equilibrium without any water movement across the membranes have been obtained using HNO_3 as the "driver" (C^+D^-) electrolyte; dipicrylamine and trioctylpropyl ammonium compounds dissolved in 2-undecanone were the membranes.

There are several possible reasons for not obtaining the maximum accumulation. The constant flow-through of the "outside" solution and its stirring may not be fast enough to remove the outwardly moving "driver" ions from the membrane interface or to replenish the RbBr at the interface. Stirring of the "inside" solution may also be insufficient to replenish the "driver" ions at the interface and remove the incoming ions that have just exchanged out of the membrane. If these processes are not fast enough, the membrane-mediated Donnan equilibrium will be based on a concentration ratio less than the nominal, bulk phase ratio and the accumulation will consequently be less than the theoretical calculated on the bulk phase ratio.

The reverse of the accumulation model in which ions move across the membrane and water does not, was found with model cells which were constructed to demonstrate osmotic flow across liquid oil membranes. Disubstituted amides

of fatty acids in which the hydrogens attached to the nitrogen have been replaced by methyl groups were used as the membranes. The amides were constrained by two porous sheets and placed between distilled water and 1.0 N KCl solutions. As an example, in the course of less than 2 hours, 6 meq. of water moved to the KCl side while only .037 meq. of Cl^- moved into the distilled water side. With longer experiments hydrostatic heads of over 20 cm were reached before limitations of the model occurred.

Significance to Biomedical Research and the Program of the Institute:

Important to all living systems are the mechanisms that regulate the permeabilities of electrolytes, non-electrolytes and water through membranes. Understanding of the complex membrane phenomena in living cells and tissues is helped by information gained from simple membrane model systems that can reproduce some of the phenomena that occur in living systems. Model studies indicate the possibility of easy explanations for phenomena that have previously been thought of as very complicated. The accumulation of electrolytes against concentration gradients in models that rely only on known physico-chemical principles reported here is one example.

The occurrence of electrical potentials across living cell membranes is widespread. How they arise and change in magnitude are subjects of extensive research which can be aided by electromotive experimentation employing the well characterized, synthetic membranes used in the project. Steady state and equilibrium potentials as well as those from fast degrading systems can be studied with a facility that is lacking with most living cell membranes. The possible transference of the information gained with the synthetic membranes to the living membranes is obvious.

Proposed Course of Project:

Some solvents solvate anions better than cations and vice versa. My experiments have indicated that cation exchanger membranes made with electron donor solvents, such as 2,6-dimethyl-4-heptanone, give higher concentration potentials than those made with electron acceptor solvents, such as 2-propyl 1-heptanol, while the reverse is true with anion exchanger membranes. A further investigation into this and the enhanced specificity of one cation over another cation that has been found with some solvents will be carried out.

Additional accumulation models will be planned in order to approach the maximum Donnan accumulation of electrolytes. Osmotic models using liquid membranes and charge-mosaic membranes made with both porous and liquid ion exchanger membrane parts will be investigated as well.

Publications:

Pages 41-51 of the following were written in cooperation with Dr. Karl Sollner:
Sollner, K.: The use of models in the study of complex effects membranes. Trans. Papal Acad. Sci., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29004-07-LCP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Photochemical reactions and their mechanisms

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

PI:	Herman Ziffer	Research Chemist	A LCP
OTHER:	Kuninobu Kabuto	Visiting Fellow	A LCP

COOPERATING UNITS (if any)

John R. Williams, Temple University

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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

We recently established that the irradiation of A-homocholest-4a(5)-ene-3-one yield two isomeric photoproducts. The major product was shown to have a cis A/B ring junction while the minor product had a trans A/B junction. When substituents on the steroid nucleus were varied less of the minor isomer formed and in one case none was observed. In order for this reaction to be useful in a synthetic scheme it is important to better understand the factors that determine the stereochemistry of the photoproducts. We therefore are currently investigating the effect of solvent and temperature on the course of the rearrangement.

PHS-6040
(12-75)

Project No.: Z01-AM-29004-07-LCP

Project Description:

In order to permit a more rational use of photochemical reactions in synthesis, a better understanding of the mechanism and stereochemical requirements of these reactions is necessary. In an earlier study of the photochemical rearrangement of A-homocholest-4a(5)-en-3-one showed that cis and trans isomeric photoproducts formed with the former stereochemistry predominating. When substituents on the steroid nucleus were varied, the predominant and in one case only photoproduct observed was the cis isomer. In a projected synthesis of a C-18 ethyl steroid we anticipated using this rearrangement and wished to direct the stereochemistry of the rearrangement to increase the yield of the trans isomer. A preliminary study of the effect of temperature and solvent demonstrated that the trans isomer was favored by conducting the irradiation at higher temperatures and in the presence of xylene. We expect to use this information to prepare some trans isomers that previously were inaccessible.

Publications:

Akiyama, T., Pedder, D., Silverton, J. V., Seeman, J. I., and Ziffer, H.: A synthesis and x-ray structure determination of the photoproducts of A-homocholestan-3-one. J. Org. Chem. 40: 3675-3680, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29005-02-LCP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Asymmetric synthesis; structure, stereochemistry and NMR

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

PI:	Herman Ziffer	Research Chemist	A LCP
OTHER:	Kuninobu Kabuto	Visiting Fellow	A LCP

COOPERATING UNITS (if any)

D. T. Gibson, University of Texas

LAB/BRANCH
Laboratory of Chemical Physics
SECTION
Section on Molecular Biophysics
INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

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

Optically active samples of the four isomeric 3-phenyl-1,2-dihydroxycyclohexanes have been prepared and their absolute stereochemistry established by oxidation to 2-phenyladipic acid of known absolute stereochemistry. Two of the alcohols were prepared by chiral reduction of cis 2-acetoxy-6-phenylcyclohexanone while the other two compounds were prepared from 3(R)-phenylcyclohexene. We have measured and compared the signs of the cd spectra for the ^1L_a transition (~220 nm) of these compounds with that predicted by the "quadrant rule" suggested by Price and Verbit. The signs of the cd curves of each isomer was correctly predicted, thus strengthening the limit experimental basis of the rule. The proton and ^{13}C nmr spectra of these compounds were measured, resonances assigned and this information used to determine the conformation present in solution.

PHS-6040
(12-75)

Project No.: Z01-AM-29005-02-LCP

Project Description:

Asymmetric Synthesis

In the course of studies establishing the absolute stereochemistry of aromatic metabolites the severe limitations associated with working with small quantities of material available from enzymatic reactions became apparent. In order to overcome these limitations we undertook a program on the asymmetric synthesis of a number of hydroaromatic alcohols. In one approach we examined the chiral synthesis of some cis and trans diols employing a lithium aluminum:ℓ-darvon (1:2) complex to reduce an α-acetoxy ketone. In a second approach we examined the use of actively fermenting yeast to stereospecifically reduce hydroaromatic ketones to optically active alcohols.

The cis and trans 1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene diols are reduction products of metabolites (bacterial and mammalian, respectively) of naphthalene. Optically active samples of each diol were prepared by chiral reduction of 1-keto-2-acetoxy-1,2,3,4-tetrahydronaphthalene with the previously described lithium aluminum-ℓ darvon (1:2) complex. The absolute stereochemistry of the cis diol was established by acetylation and hydrogenolysis to the known (-)-2(S)-acetoxy-1,2,3,4-tetrahydronaphthalene, while the absolute stereochemistry of the trans diol was known. The quantities of material and optical purity (20% e.e. for the cis diol and 62% e.e. for the trans diol) obtained via this reduction suggests that this method can be used to prepare material for chemical transformation that was not previously available from enzymatic studies.

The alcohols obtained by microbiological reduction of the following ketones:

were isolated. The absolute stereochemistries of these alcohols were established by acetylation, ozonolysis, esterification, and isolation of α-acetoxy-dimethyl adipate and α-acetoxy-dimethylglutarate of established absolute stereochemistry. Each alcohol was found to have an (S) configuration, demonstrating that the enzyme(s) responsible for this reduction is product stereospecific. The method thus allows one to reduce an α-keto hydroaromatic compound to an optically active alcohol of predictable absolute stereochemistry. Further studies using this reduction are in progress.

Structure, Stereochemistry and NMR

Optically active samples of the four isomeric 3-phenyl-1,2-dihydroxy-cyclohexanes have been prepared and their absolute stereochemistry

established by oxidation to 2-phenyladipic acid of known absolute stereochemistry. Two of the alcohols were prepared by chiral reduction of cis 2-acetoxy-6-phenylcyclohexanone while the other two compounds were prepared from 3(R)-phenylcyclohexene. We have measured and compared the signs of the cd spectra for the ^1L_a transition ($\sim$220 nm) of these compounds with that predicted by the "quadrant rule" suggested by Price and Verbit. The signs of the cd curves of each isomer was correctly predicted, thus strengthening the limit experimental basis of the rule. The proton and ^{13}C nmr spectra of these compounds were measured, resonances assigned and this information used to determine the conformation present in solution.

Publications:

Ziffer, H. and Gibson, D. T.: Relative and absolute stereochemistry of diols obtained from microbial oxidation of 3-methylcyclohexene. Tetrahedron Letters: 2137-2138, 1975.

Kabuto, K. and Ziffer, H.: Asymmetric synthesis and absolute stereochemistry of some cis and trans diols. J. Org. Chem. 40: 3467, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29006-06-LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The relationship between the nuclear and electronic configurations of molecules and their spectroscopic and dynamic properties.

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

PI:	Elliot Charney	Research Chemist	A LCP
OTHER:	Joan Rosenfield	Staff Fellow	A LCP
	Holly Ho Chen	Guest Worker	A LCP

COOPERATING UNITS (if any)

Kiwamu Yamaoka, University of Hiroshima, Japan
W. B. Whalley, University of London, England

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Spectroscopy and Structure

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3	1.5

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

A. Molecular chiral phenomena are being examined by quantum mechanical calculations, (CNDO/S and CNDO/2), in organic compounds containing intrinsically disymmetric conjugated systems.

B. Macromolecular structure and dynamics of large biological polymers in particular polynucleotides, are being studied by electric-field induced dichroism and birefringence methods.

PHS-6040
(12-75)

Project No: Z01-AM-29006-06-LCP

Project Description:

This report covers projects in two different but allied fields: (1) Molecular chiral phenomena and (2) macromolecular structure and dynamics. The object of the first project is to try to increase our understanding of the nature of molecular chiral phenomena in general and in particular to see the extent to which quantum mechanical interpretation of molecular states provide a detailed explanation of the chirality of small molecules or individual chromophores. The objectives of the investigations into macromolecular structure and dynamics are to determine the molecular structure of polymers, biological polymers in particular, in determining their physical properties - and the converse as well. The special experimental methods employed have been described in previous reports and the theoretical methods are standard in the field.

1. Molecular Chiral Phenomena

a) Access to new experimental data on the optical activity of molecules containing the diene group, - CH=CH-CH=CH -, has led us to examine theoretically the chiral effect of substitutents in molecules containing this group. The fascinating result that hydrogen substituents allylic to the end carbons can give vastly different optical activity than allylic methyl substituents, a fact confirmed by the available experimental data, has led us to consider the possibility that this may be one of the more sensitive probes for testing some of the assumptions (the nature of the electronic wavefunctions and the degree to which various interchange interactions may be neglected) of quantum mechanical calculations.

b) The work on α-diketones reported as started in the last annual report has been temporarily set aside in order to concentrate on the dienes described above.

c) The monograph described in the last annual report is nearing completion.

2. Macromolecular Structure and Dynamics

The orientation of large polymeric molecules in pulsed electric fields and their disorientation when the fluid is turned off can be studied using monochromatic polarized light. These experiments are designed to obtain several types of information, including the molecular conformation or structure in solution (from the dichroism, which is the difference between the changes in extinction of the light polarized parallel and perpendicular to the electronic field when the molecules are completely orientated in the field) and the dynamics of intramolecular reorganization and a rotational disorientation (from the decay of the dichroism at the end of the field pulse).

The principal project in this area has been a study of polyribose cytidilic acid, poly(r) C. We have measured the field dependence and wavelength dependence of the birefringence and dichroism of poly(r) C and found among

Other things an optical transition long suspected on the long wavelength edge of the main $\pi \rightarrow \pi^*$ optical transition at 270 nm. This is apparently the transition identified as an n-π^* transition by Rich and Kasha [J. Amer. Chem. Soc. 82, 6197 (1960)] on a film of polycytidilic acid which has been the subject of controversy ever since. Further analysis of the data for structural parameters of poly(r) C in solution is in process.

Proposed Course of the Project:

In addition to the completion of the projects described above as underway, it is proposed to continue with several new or related investigations of molecular dynamics by electric dichroism and/or birefringence. In particular, we expect to examine in detail the dichroism and relaxation of polypeptides in which a freely rotating link is incorporated approximately in the center of the longer polymer. It is our expectation that in the presence of the electric field these molecules will orient as though they were a continuous rod. It remains to be seen whether these expectations will be correct and to examine in detail the relaxation phenomena which have a direct bearing on earlier investigation of the relaxation of polyriboseadenylic acid.

Publications:

Rosenfield, J. S.: Magnetic rotational strengths of higher states in benzene: Evidence for the existence of an out-of-plane transition. Chem. Phys. Letters 39: 391-394, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29007-05-LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Structure and interaction of paramagnetic biomolecules

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

PI:	Hideo Kon	Research Chemist	A LCP
OTHER:	Mitsuo Sato	Visiting Fellow	A LCP
	Amy S. Rispin	Guest Worker	A LCP

COOPERATING UNITS (if any)
D. W. Nebert (CH-DPB), E. C. Weinbach (I-LPD)
J. V. Silverton (H-IRCH)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Spectroscopy and Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

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

Major effort of our work this year is centered around the investigation of the electronic structure and the reaction of cytochrome P-450 using a model system as well as mammalian liver microsome. The model system for Fe(III) in P-450, which we reported last year, does not involve any sulfur containing ligand, yet does simulate nicely the characteristic epr (electron paramagnetic resonance) behavior of Fe(III) in native P-450. In order to study the details of the electronic structure of Fe(III), the refinement in the method of analysis of epr data is made this year by retaining all the quartic terms in the spin Hamiltonian. On the reaction side, the benzo[a]pyrene-microsome system is of unique interest in that the reaction yields some free radical. By using $^{17}O_2$-nitrogen mixture in place of ordinary air, we have proved that the reaction is mediated by P-450, the radical is an oxyradical, and the source of oxygen is atmospheric. The free radical signal, however, is mostly due to the non-enzymatic air oxidation of some precursor formed during the incubation. Other systems studied include Co(II)tetraphenylporphine and non-heme iron protein in ENTAMOEBA HISTOLYTICA.

PHS-6040
(12-75)

Project No: Z01-AM-29007-05-LCP

Project Description:

The fundamental objective of this project is to clarify the structure-function relationship of the paramagnetic centers existing in various biological systems. Of the two categories of the paramagnetic molecules, i.e. organic or inorganic free radicals and the transition metal ions, more emphasis has been placed in this project on the transition metal containing biomolecules and the model systems.

The facilities include an electron paramagnetic resonance (epr) spectrometer, a Faraday balance to measure the static magnetic susceptibility, and an electron-nuclear double resonance (endor) spectrometer (construction underway). Since the analysis of the observed results often requires a higher order pertubation method and matrix diagonalization, the use of the digital computor is almost indispensable.

Major Findings:

1. (With Dr. Rispin)

It has been known that an epr signal of a free radical is observed in the benzene extract of the reaction mixture (liver microsome, benzo[a]pyrene, cofactor, in air). By replacing air with $^{17}O_2$ containing nitrogen, we proved unequivocally that the free radical is an oxyradical, and oxygen incorporation to benzo[a]pyrene is mediated by cytochrome P-450 and that some precursor, not the free radical, is the immediate product of this reaction. The free radical signal we observe in the final benzene extract is mostly the result of non-enzymatic air oxidation of the precursor.

2. (With Dr. Sato) Continuation from #1 and #2 in 1975 Report.

In order to determine the d-orbital energy levels of Fe(III) in the highly distorted rhombic environment, such as in cytochrome P-450, a refinement in theoretical analysis is made by including all the quartic terms in the spin Hamiltonian. Increased number of adjustable parameters makes the unique determination difficult. However, a method is devised to fit most of the parameters and confine the rest in very narrow ranges by extensive use of the crystal field perturbation theory. The method is applied to analyse the temperature dependence data of the model compounds for P-450. Extensive numerical tabulations are made of the necessary functions to facilitate such analysis in general.

3. (With Drs. Sato, Akoh, Tasaki, Kabuto, and Silverton) Continuation of #3 in 1975 Report).

The observed magnetic susceptibility of the epr absent paramagnetic species of Co(II)tetraphenylporphine is found to fit very well the Ising model with $g_z = 6$, $g_{x,y} \sim 0$ the result which can not be explained on the basis of hitherto assumed low-spin configuration of this complex. At the same time the x-ray structural analysis of this species revealed a no-planar D_{2d}

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structure with the bonding nitrogen lone-pair orbitals pointing off the Co-N direction, thus strongly enforcing a high-spin interpretation which is reported for the first time in Co(II) porphyrin complexes.

4. (With Dr. Weinbach) Continuation of collaborative effort #4 in 1975 Report.

The epr signal from the iron-sulfur protein in ENTAMOEBA HISTOLYTICA is unusually narrow (peak-to-peak width = 25 G) and quite symmetrical. The question arises whether the signal is indeed associated with iron (rather than a free radical). The comparison of the signal linewidth of the preparations grown in ^{57}Fe rich medium and the one from ordinary culture gave positive evidence for the involvement of iron. The signal saturation behavior at low temperature also supported the conclusion.

Short term future plan:

Continued effort will be made in the study of Fe-porphyrin systems with rhombic distortion in the hope to clarify the mechanism of the distortion. As the construction of endor spectrometer progresses the study of NO-heme-protein complex may be resumed. The use of the newly rebuilt Faraday balance is expected to start shortly.

Publications:

Sato, M. and Kon, H.: Epr studies on the high spin Fe(III) tetraphenylporphine with rhombic character. Determination of zero-field splitting parameters from the middle Kramers transition. Chem. Phys. 12: 199-211, 1976.

Rispin, A. S., Kon, H. and Nebert, D. W.: Electron spin resonance study of oxygen-17 enriched oxybenzo[a]pyrene radical. Mol. Pharm. (in press).

Sato, M., Kon, H., Akoh, H., Tasaki, A., Kabuto, C. and Silverton, J. V.: Anomalous magnetic properties of tetraphenylporphinato Co(II) complex in the solid state. Chem. Phys. (in press).

Berzofsky, J. A., Schechter, A. N. and Kon, H.: Does Freund's adjuvant denature protein antigens? EPR studies of emulsified hemoglobin. J. Immunol. 115: 270-272, 1976.

Weinbach, E. C., Diamond, L. S., Claggett, C. E. and Kon, H.: Iron-sulfur proteins of ENTAMOEBA HISTOLYTICA. J. Parasitol. 62: 127-128, 1976.

Weinbach, E. C., Harlow, D. R., Takeuchi, T., Diamon, L. S., Claggett, C. E. and Kon, H.: Aerobic metabolism of ENTAMOEBA HISTOLYTICA: Facts and fallacies. Arch. Invest. Med. (MEXICO) (in press).

REPORTED IN PRESS - 1975

Sato, M. and Kon, H.: The high spin Fe(III)tetraphenylporphine with unusually large rhombic character. Inorg. Chem. 14: 2016-2018, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29008-05-LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Electronic and molecular structural investigations

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

PI: Ruth McDiarmid Research Chemist A LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics
SECTION
Spectroscopy and Structure
INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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

During the last year 2 projects were completed: (1) The ultraviolet absorption spectra of trans-1,3-butadiene, 1,1,4,4-trans-1,3-butadiene-d_4, and trans-1,3-butadiene-d_6 between 2300 and 1350 A were recorded and analyzed. Four Rydberg series were identified (δ = 0.087, 0.21, 0.42, and 0.67). A transition-by-transition comparison of this analysis with those in the literature shows that several of the previously assigned transitions are misidentified. A continuum originating around 1450 Å is reported.

(2) The absorption spectrum of gaseous UF_6 was recorded from 1200-4200 Å. (83 300-23 800 cm^{-1}). 8 electronic transitions were observed with intensities ranging from 8-20 000 l/mole·cm. Based on correlations between this spectrum and those of UF_6^-, UCl_6^-, MoF_6 and WF_6 and the photoelectron spectrum of UF_6, the experimental spectrum was assigned.

PHS-6040

Current Project:

The current project is the analysis of the electronic spectrum of isobutene. The analysis of this molecule, when completed, in conjunction with the already completed analyses of cis- and trans-butene [R. McDiarmid, J. Chem. Phys. 50, 2328 (1969)] will enable conclusions to be drawn concerning the effect(s) of substitution on the quantum defects of related ethylenic molecules.

It is also hoped that the contour of the 1785 Å transition of isobutene will provide sufficient detail for an assignment of the band-type. This assignment, in conjunction with the observed quantum defects of the other transitions, will enable us to test a set of rules proposed for assigning specific Rydberg series within the atomic Rydberg s-, p- and d- classifications.

Significance to Biomedical Research and the Program of the Institute:

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.

Of isobutene investigation: The analysis of this spectrum completes the series of substituted ethylenes. Hopefully, rules can now be generated, which can be extrapolated to larger molecules, and which will predict the spectral changes ensuing on simple changes in the position(s) of chemical substituents.

Proposed Course of Project:

1. This year we intend to investigate the ultraviolet absorption spectra of several unstable molecules of interest. Initially we hope to generate, observe and analyze $H_2C{=}S$ and HN=NH.

2. A more formal investigation of the effect of electronic excitation on torsional barriers will be initiated.

3. We plan to return to the previous study of saturated ethers. This time, the investigation will encompass heavier ethers [$(CH_3)_2Se$, $(CH_3)_2$ Te] in an attempt to support our earlier analysis of the spectrum of $(CH_3)_2$ S.

Publications:

McDiarmid, R.: On the ultraviolet spectrum of trans-1,3-butadiene. J. Chem. Phys. 64: 514, 1976.

McDiarmid, R.: Assignments in the electronic spectrum of UF_6. J. Chem. Phys., in press.

Project No: Z01-AM-29008-05-LCP

REPORTED IN PRESS - 1975

McDiarmid, R.: On the forbidden 2000 Å transition of trans-1,3-butadiene. Chem. Phys. Letters, 34: 130, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29009-03-LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies on sickle cell disease

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

PI:	William A. Eaton	Senior Surgeon	A LCP
OTHER:	James Hofrichter	Staff Fellow	A LCP
	Philip D. Ross	Research Chemist	A LMB

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Spectroscopy and Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

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

The thermodynamics and kinetics of the gelation of sickle cell hemoglobin are being investigated in order to develop an inhibitor of the reaction that could be used as a therapeutic agent.

PHS-6040
(12-75)

Project No.: Z01-AM-29009-03-LCP

Objectives:

The major aim of this work is to develop agents for the treatment of sickle cell disease through physico-chemical studies on the gelation of hemoglobin S.

Methods Employed:

We have developed a variety of techniques to study the gelation reaction, including scanning microcalorimetry, optical birefringence, light scattering, water proton nmr linewidths, and sedimentation methods.

Major Findings:

We have continued our exploration of the kinetics and thermodynamics of sickle cell hemoglobin gelation (polymerization). Four different physical properties have been used to monitor the kinetic progress curves - turbidity, birefringence, heat absorption, and water proton magnetic resonance linewidths. From studies on identical samples at different temperatures we have established that all four techniques see the onset of gelation simultaneously. This result is consistent with our earlier hypothesis that the rate of gelation is limited by the rate of nucleation for individual polymers. It has also permitted us to study the influence of physiological parameters on the rate of gelation using the single most efficient technique, which is the turbidometric method.

By measuring the delay time of gelation and the solubility on identical samples, we have confirmed the supersaturation equation which was used to predict gelation delay times under in vivo conditions. Of particular importance is the finding that the supersaturation equation is obeyed in the presence of carbon monoxide, strongly indicating that sickle cell hemoglobin solutions partially saturated with oxygen gel by the same basic mechanism that we have proposed for completely deoxygenated solutions.

A strong correlation has been found between the predicted gelation delay time for mixtures of hemoglobin S with hemoglobins A and F and clinical severity, strongly indicating that the gelation delay time may be the primary determinant of clinical severity in sickle cell disease. If this is the case, then it is possible to estimate that about a 100-fold increase in gelation delay time in vivo will be necessary to produce a clinical effect. This provides a quantitative criterion for evaluating the potential usefulness of various proposed therapeutic agents. Urea, for example, at concentrations that can be achieved in vivo lengthens the delay time by only a factor of about 2, suggesting that it would not be a useful drug.

Significance to Biomedical Research and the Program of the Institute:

Our kinetic study constitutes a totally new approach to understanding the pathophysiology of sickle cell disease and to the development of therapeutic measures.

Both the theory and methods developed in this investigation should contribute to a general understanding of macromolecular assembly processes.

Proposed Course of Project:

We shall measure the kinetics and thermodynamics of sickle cell hemoglobin gelation in solutions partially saturated with oxygen or carbon monoxide. Methods will be developed to study the reaction on much shorter time scales, both for the sake of collecting kinetic data more efficiently, but also to study the faster rates that take place in vivo. To mimic the continuous desaturation of hemoglobin that takes place inside a capillary, we will control and continuously change the saturation with carbon monoxide with a photodissociating light beam.

We shall undertake a small scale screening program of materials that could be potential therapeutic agents. The agents will be chosen from those whose toxicity in humans is already well-established, since it is the accumulation of toxicity data that will most probably be the rate-limiting step in finding an effective drug for sickle cell disease.

In collaboration with clinical investigators we shall attempt to establish a correlation between gelation delay times and clinical severity in patients with homozygous S disease.

Publications:

Eaton, W. A., Hofrichter, J. and Ross, P. D.: Delay time of gelation: A possible determinant of clinical severity in sickle cell disease. Blood, 47: 621-627, 1976.

Eaton, W. A., Hofrichter, J., Ross, P. D., Tschudin, R. G. and Becker, E. D.: Comparison of sickle cell hemoglobin gelation kinetics measured by nmr and optical methods. Biochem. Biophy. Res. Comm. 69: 538-547, 1976.

Hofrichter, J., Ross, P. D. and Eaton, William A.: Supersaturation of sickle cell hemoglobin solutions. Proc. Nat. Acad. Sci., in press.

Hofrichter, J., Ross, P. D. and Eaton, William A.: A thermodynamic and kinetic description of the gelation of deoxyhemoglobin S, in Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease, in press.

REPORTED IN PRESS - 1975

Ross, P. D., Hofrichter, J. and Eaton, W. A.: Calorimetric and optical characterization of sickle cell hemoglobin gelation. Hofrichter, J.: Appendix: Theory of the optical birefringence of sickle cell hemoglobin gels. J. Mol. Biol. 96: 239-253, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29010-04-LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Conformation and electronic structure of biological molecules

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

PI:	William A. Eaton	Senior Surgeon	A LCP
OTHER:	Louise K. Hanson	Guest Worker	A LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Spectroscopy and Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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

The electronic structure of heme proteins is being investigated using a combined theoretical and experimental approach, that includes extended Hückel molecular orbital calculations and various electronic spectroscopic measurements.

PHS-6040

Project No.: Z01-AM-29010-04-LCP

Objectives:

The general aim of the research is to relate the stereochemistry and electronic structure of biological molecules, particularly proteins with chromophoric prosthetic groups, to their biochemical reactivity and function.

Methods Employed:

In addition to conventional biochemical and spectroscopic methods, techniques have been developed for measuring anisotropic optical properties on microscopic samples. Dr. Hofrichter has designed and constructed a general phase modulation microspectrophotometer which is capable of measuring linear dichroism, circular dichroism, linear birefringence, and circular birefringence with sensitivity comparable to commerical instruments used for macroscopic samples. The instrument also measures direct polarized absorption, and is interfaced to our Honeywell 516 computer.

In order to interpret the electronic spectra in terms of the orbital promotion mechanism responsible for observed electronic transitions we have used the self consistent charge, extended HÜckel LCAO-molecular procedure developed by Gouterman and coworkers at the University of Washington.

Major Findings:

a. Electronic structure of oxyhemoglobin.

Using the extended HÜckel theory, we have calculated the molecular orbitals for an imidzaole-iron-porphyrin oxygen complex using the coordinates for the positions of the nuclei from small-molecule X-ray studies. With the resulting orbital energy level diagram we are able to make tentative specific assignments for the orbitals that are responsible for the electronic transitions of oxyhemoglobin. The assignments are consistent with the observed frequencies, polarizations, natural circular dichroism, and magnetic circular dichroism results. The self-consistency of the experimental and theoretical results suggests the extended HÜckel model is providing an excellent first approximation to the description of the heme-oxygen bonding in oxyhemoglobin. The most outstanding feature of the theoretical results is the creation of heavily mixed iron and oxygen orbitals, suggesting that pi bonding makes an important contribution to the stability of oxyhemoglobin.

b. Cytochrome P-450

We have continued our spectral studies, primarily the polarized absorption spectra of single crystals, of the monoxygenase hemoprotein cytochrome P450 on substrate-bound purified material from the bacterium Pseudomonas putida. Cytochrome P450 has been shown recently both by our work and the work of porphyrin chemists to most probably contain a cysteine ligand to the heme. In order to interpret the unusual spectral features exhibited by this protein, we have performed extended HÜckel molecular orbital calculations on heme model compounds with methyl mercaptide and methyl mercaptan as axial ligands. These

calculations have been very successful in explaining the electronic spectra and thereby the electronic structure of P450 in various states of its enzymatic cycle, namely the oxidized, reduced, CO-reduced, and O_2-reduced forms. The striking anomalous features of the CO-reduced form and the new bands we observe in the oxidized form can be attributed to the presence of lone pair mercaptide (RS^-) cysteine orbitals in the valence electronic region of the heme. On the other hand, the reduced and O_2 reduced forms, which exhibit more "normal" spectra, probably have mercaptan (RSH) cysteine as an axial ligand. In this latter case, the lone pair sulfur orbitals are too low in energy to appreciably affect the valence electronic structure of the heme.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the electronic structure of the active sites of proteins should lead to a much deeper understanding of how these molecules perform their biological function.

The techniques developed in our investigations may be applied to a wide variety of problems in protein structure and chemistry.

Proposed Course of Project:

Because there are so many possible electronic transitions a final step in making specific spectroscopic assignments is to calculate magnetic and electric dipole transition moments for all possible transitions in the frequency range of our measurements. These should not only make our current assignments for oxyhemoglobin more definite, but they should also provide the necessary background for using a similar combined experimental and theoretical approach for understanding the more complex electronic spectrum and electronic structure deoxyhemoglobin and other heme protein complexes.

Publications:

Hanson, L. K., Eaton, W. A., Sligar, S. G., Gunsalus, I. C., Gouterman, M. and Connell, C. R.: Origin of the anomalous soret spectra of carboxy cytochrome P450. J. Amer. Chem. Soc. 98: 2672-2674, 1976.

Eaton, W. A., Hofrichter, J., Hanson, L. K. and Makinen, M. W.: Single crystal optical spectroscopy of hemolgobin. Proceedings of the Taniguchi International Symposium in Biophysics on Metalloprotein Studies Utilizing Paramagnetic Effects of the Metal Ions as Probes, in press.

Hofrichter, J. and Eaton, W. A.: Linear dichroism of biological chromophores. Ann. Rev. Biophys. Bioeng. 5: in press.

REPORTED IN PRESS - 1975

Eaton, W. A., Hofrichter, J., Makinen, M. W., Andersen, R. D. and Ludwig, M. L.: Optical spectra and electronic structure of FMN in flavodoxin crystals. Biochemistry 14: 2146-2151, 1975.

Project No.: Z01-AM-29010-04-LCP

Padlan, E. A., Eaton, W. A. and Yonetani, T.: A crystallographic study of deoxy cobalt(II)mesoporphyrin IX myoglobin. J. Biol. Chem. 250: 7069-7073, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29011-05-LCP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
The Physics and Chemistry of Photoreception

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

PI:	William A. Hagins	Medical Officer	A LCP
OTHER:	W. E. Robinson	Senior Staff Fellow	A LCP
	S. Yoshikami	Guest Worker	A LCP
	F. M. Hagins	Guest Worker	A LCP
	H. RÜppel	Guest Worker	A LCP
	J. Meisner	Laboratory Technician-- Summer Employee July-Aug. 1975 and 23 May-1 July 1976	A LCP

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Membrane Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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

An investigation of the mechanism of visual excitation in vertebrates.

PHS-6040
(12-75)

Project No.: Z01-AM-29011-05-LCP

Project Description:

The central aim of this project is to describe the chemical and electrical steps that connect absorption of a photon of visible light in a photoreceptor cell with the transmission of a sensory signal across its synapse with the distal neurons of the brain. Previous work by our group on the rod and cone cells of vertebrate retinas have established that an essential part of their excitatory machinery is a large ionic current that circulates through these cells in darkness. This "dark current" enters the plasma membrane covering the light absorbing outer segment of a rod or cone, passes down the cytoplasm of the cell and emerges into the extra-cellular space through the plasma membrane of its more proximal parts. Light absorbed in the outer segment quickly and reversibly reduces the dark current, thus hyperpolarizing the cell and probably changing the rate of release of a neurosensory transmitter substance at its synapse with deeper lying neurons.

During the past year our work has concentrated on two questions: (1) What is the identity of the excitatory chemical substance that is released intracellularly when light is absorbed by the photopigment, rhodopsin? (2) What biochemical machinery provides energy for amplification of the light responses of vertebrate rods and cones?

Question 1) There is now much evidence that light causes a diffusible substance to be released intracellularly in the outer segments of retinal rods. This substance must spread from points where light quanta are absorbed in rhodopsin molecules to the surface membrane of the rod cell, where it causes the dark current to be temporarily reduced by interfering with the movement of current-carrying Na^+ ions through sites in the membrane. Theoretical arguments, (Yoshikami & Hagins, 1976) indicate that each absorbed light quantum must release tens or hundreds of transmitter molecules in the rod cytoplasm. But to measure the actual number released requires that they be chemically identified.

Indirect evidence from our laboratory strongly suggests that the transmitter may be free Ca^{++} ions. If so, introduction of calcium ion buffers into the rod cytoplasm should have the effect of desensitizing the cells to dim light in a characteristic way, because some of the Ca^{++} ions released by light should be trapped by the buffer and be unavailable for interacting with the Na^+ sites in the plasm membrane. Direct attempts to introduce Ca^{++} ion buffers into rods have not been successful; cell membranes are almost totally impermeable to those, like EDTA and EGTA, that have the strong Ca^{++} binding properties needed for intracellular buffering experiments. However, a new technique has been used to introduce such substances by the method of fusion of buffer-bearing phospholipid vesicles with rod plasma membranes. By including fluorescent dye tracers along with the Ca^{++} buffers it has been possible to show that more than 10 micromolar concentrations of Ca^{++} buffers can be attained in rod cytoplasm. The predicted effects on the light responses of the cells have been observed. We thus conclude that a) the excitatory transmitter of visual excitation in vertebrate rods is a divalent metal ion that

is not Mg^{++}. b) Many ions, hundreds or thousands, are released for each light quantum absorbed. Because Ca^{++} is the only divalent metal abundant enough in rod outer segments for such effects, we believe its role as the excitatory transmitter has been established.

Question 2) Storage of Ca^{++} in darkness and its release in light require more energy than is available in the light quanta themselves. This energy must come from the metabolism of the cells, probably from the hydrolysis of nucleoside triphosphates. Studies of ATPases of purified rod outer segments indicate a small but inseparable ATPase activity in them. In addition, there's a large ATP consumption amounting to at least 0.1 mol per mol of rhodopsin when isolated rods are illuminated. This ATP consumption does not result in phosphorylation of rhodopsin and it is often complete in less than 15 sec. The biochemistry of the reaction is now under study.

Our studies indicate that retinal rods and cones share much of the electrical and biochemical machinery of neurons. Their most conspicuous difference is their huge dark current, which turns over their entire content of univalent cations in about 1-5 minutes and requires a rate of metabolism as high as that of any known tissue. Light, in controlling this current, not only acts as a sensory stimulus but also must have a great effect on the metabolic load that rods and cones carry. A clear understanding of the metabolism of these cells is therefore important in handling certain retinal vascular diseases and retinal detachment. The scientific importance of understanding the mechanism of visual excitation speaks for itself.

Publications:

Hagins, W. A., Robinson, W. E. and Yoshikami, S.: Ionic Aspects of Excitation in Rod Outer Segments, Energy Transformation in Biological Systems. Ciba Foundation Sym. 31. Amsterdam, North Holland, Elsevier, 1975, pp. 169-189.

Hagins, W. A. and Yoshikami, S.: Ionic Mechanisms in Excitation of Photoreceptors, Annals of the New York Academy of Sciences, 264, 314-325, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29012-06-LCP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The influence of molecular structure on chemical and biological properties

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

PI:	Norman E. Sharpless	Research Chemist	A LCP
OTHER:	Ralph G. Adams	Research Physicist	A LCP
	Frederick S. Brackett	Scientist Emeritus	A LCP
	William H. Jennings	Research Physicist	A LCP
	Ulrich Weiss	Research Chemist	A LCP

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Membrane Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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

The tripeptide ℓ-α-acetyl lysyl-l-alanyl-l-phenylalanine methyl ester is being used as a paradigm of the visual protein opsin, to gain insight into the visual process, especially with regard to the origin and interpretation of the 500 nanometer absorption peak in rhodopsin. We feel that this peak arises from a charge-transfer interaction between the retinene chain and the phenyl ring. Retinene is known to form several complexes interpretable as charge transfer and one finding is that the reaction of retinene with antimony trichloride results in sufficient unpairing of an electron to an ESR signal. Retinal reacts with this tripeptide to parallel its spectral activity with phospholipids and perhaps opsin. An absorption at 480 nanometers may be equivalent to the 500 nanometer peak in rhodopsin and be a charge transfer band. Spectral, optical rotatory dispersion and circular dichroism investigations have been made. A program for calculating molecular geometries by the strain energy minimization method has been finally debugged. Since in its original form this program handled only carbon, hydrogen and oxygen, it was necessary to calculate many more additional parameters (bond moments, bending force constants etc) involving atoms and bonds important in biologically active molecules.

Project No.: ZO1-AM-29012-06-LCP

Project Description:

This project investigates molecular and sub-molecular factors such as structures, quantum parameters and physical properties in relation to biological and pharmacological functions of biologically important substances.

Therapeutic interference. Methods of protecting the primary amino groups of acridine drugs so that the spin label can be attached to the tertiary nitrogen atom are being investigated. The most promising at present seems to be t-butyloxycarbonyl derivatives, which apparently have not previously been applied to aromatic amines. It is extremely difficult to force the second protective group on proflavine, but a recently synthesized product of very high melting point (>340°) may be the desired product.

The reactions of aromatic amines with salts of thiocyanoacetic gives, instead of the expected amine salt, an N-aryl-α-carbamyl-mercaptoacetamide. The mechanism of this reaction is unknown, but it may involve a cyclic intermediate. The reaction could not be sterically hindered using a blocked amine and molecular orbital calculations indicate that thiocyanoacetic acid may be only marginally stable with respect to the postulated cyclic intermediate.

Molecular orbital calculations have been carried out to gain insight into structure-activity relationships of biologically important molecules (phenols, chlorinated hydrocarbons). The most promising factor seems to be the energy levels of the molecules rather than the total energies or electron densities.

Further Plans:

Plans are underway to further the tripeptide-retinene complex, investigating the reaction with other retinene isomers as well as investigating medium effects.

The spin labeling research will be continued. One additional approach will be the formation of photoinduced radicals of acridine nitroxides.

Publications:

Rice, Kenner C., Sharpless, Norman E., Weiss, Ulrich and Highet, Robert J., "The Reaction of Dimethyl-β-ketoglutanate with 1,2-dicarbonyl compounds. III. Exo-tetracyclo [5.5.1.$0^{2,6}$.$0^{10,13}$] tridecane-4,8,12-trione." Tetrahedron Letters, 44, 3763-3766 (1975).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29013-05-LCP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The physics and chemistry of the visual pigments

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

PI:	Ralph G. Adams	Research Physicist	A LCP
	Norman E. Sharpless	Research Chemist	A LCP
OTHER:	None		

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

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

Model systems to approximate rhodopsin are devised and tested with differing combinations of phospholipid components and/or peptide fragments. A tripeptide postulated to be the active center of rhodopsin has been synthesized and is under investigation as a paradigm of the chromophoric attachment.

Project No.: Z01-AM-29013-05-LCP

Project Description:

This project is to elucidate the structure and action of the visual pigment (rhodopsin) chromophore, with the aim of constructing an in vitro system behaving similarly to the in vivo systems.

Preliminary investigations have shown that the visual pigment chromophore, retinal, can form π-π complexes with either ethanolamine phosphoglyceride (EPG) or phenylalanine, both of which are components of the visual system. Since only three amino acids are known to be intimately associated with retinal after isolation, we have used this fact to postulate that the ultimate chromophore is retinal bound to a three amino acid peptide lysyl-alanyl-phenylalanine, by both an imino bond and a π-π interaction.

Nine months ago an adequate (3 g.) supply of the tripeptide was obtained from Yeda Chemicals in Israel. We have characterized it by NMR, IR, ORD, UV spectra, its melting point and thin layer chromatographic homogeneity and performance in a complex with retinal. Comparison with the material I synthesized demonstrate the two are identical.

The complex formed with retinal in chloroform methanol (2:1) by the tripeptide differs from that previously made with ethanolamine phosphoglyceride (EPG) only that its absorption maximum is at 480 nm instead of 500 nm. The kinetics are not completely determined but appear to be not much different. Temperature studies are consistent with the concept of a charge transfer (π-π) mechanism as proposed.

The 480 nm complex appears to be more light sensitive (unstable) than the 500 nm formed with EPG which is being currently explored.

The spectral characteristics and composition (all trans retinal) suggest that the complex may be identical to an intermediate of the photobleaching process of mammalian rhodopsin, meta rhodopsin II, and that use of 11-cis retinal may produce the rhodopsin chromophore--this is currently under investigation.

Most of the data currently and previously generated is accumulated on the Honeywell 516 computer and stored on tapes. Complete analysis has not been finished. Our new program for computing optimal configurations is not completely debugged but is expected to be operational shortly (see Sharpless).

Future Work:

Literature reports indicate that all the retinal isomers may be involved in the visual excitation process and therefore should be included in follow up of current experiments.

Obviously important in the visual system is the milieu of lipid and protein wherein the visual pigment finds its place--we propose to try a series of different solutions normally occurring within the retinal elements. If some expertise can be recruited we may try artificial membranes (lipid).

Since these procedures are all slow and painstaking--a years work is thus outlined.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1-AM-29014-16-LCP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effects of environmental stress (hypoxia, exercise, smoke) on animals.

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

PI:	Paul D. Altland	Research Physiologist	A LCP
OTHER:	Howard F. Brubach	Biologist	A LCP
	Milton G. Parker	Biological Lab Technician	A LCP

COOPERATING UNITS (if any)
Barnett Rattner (Pre-doctoral Guest Worker)
Department of Zoology, University of Maryland

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

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

In studies on the basic mechanisms underlying the occurrence of hyperbilirubinemia at altitude it was found that the main factors are increased destruction of erythrocytes associated with severe polycythemia (hematocrit above 68%), increased intravascular hemolysis due to increased blood viscosity, and inability of the liver to handle increased levels of bilirubin. Studies are being conducted on physiological and biochemical changes after exposure to cigarette smoke in rats at rest and after exercise. Studies are being conducted on the effects of hypoxia on reproductive function prior to blastocyst implantation and on male reproductive function in mice.

Project No.: Z01-AM-29014-16-LCP

Project Description:

To determine physiological, biochemical and pathological changes which occur following exposure to altitude (hypoxia), exercise and other environmental stresses. Altitude exposures were conducted in a small decompression chamber. Exercise was performed in an exercise wheel located in a sealed chamber.

Bilirubinemia is known to occur in men exposed to high altitude, but the causative factors are not clearly established. Altered conjugation and delayed excretion of pigment by the liver have been considered to be important factors, but the possible effects of severe polycythemia, altered erythrocyte fragility and intravascular hemolysis have not been investigated before and the rate of development of bilirubinemia is not known. In a study of rats during acclimatization to 18,000 ft simulated altitude (5486 m) it was found that both continuous and intermittent (4 hr/day) exposures induced significant elevations in serum bilirubin within 4 to 6 weeks. The elevations occurred only after the development of severe polycythemia (hematocrit 68.5%, blood hemoglobin 21.6 g/100 ml). An increase in intravascular hemolysis was found after 2 weeks intermittent exposure and after 4 weeks continuous exposure to 18,000 ft. No change in erythrocyte fragility was found in any of the rats to account for increased intravascular hemolysis. No liver pathology was observed in rats exposed to 18,000 ft. It is significant that bilirubinemia did not occur until the polycythemia was severe (hematocrit above 68%). The causative factors appeared to be the increased destruction of erythrocytes associated with the polycythemia, the increased intravascular hemolysis due to increased blood viscosity, and the inability of the liver to handle increased levels of bilirubin.

Experiments are being conducted on the effects of hypoxia on reproductive function prior to placentation in order to determine the levels of hypoxia (8 - 21% O_2) which impairs blastocyst development and implantation. Studies are also being conducted to determine the effects of different degrees of hypoxia on reproductive function in the male mouse, with emphasis upon serum hormone levels and morphological alterations. Preliminary studies have indicated that the reproductive system of the male mouse possesses an unusually high resistance to hypoxia. Studies are also being continued on the effects of environmental tobacco smoke, nicotine free smoke, nicotine injections and different concentrations of carbon monoxide upon the physiological and biochemical changes in rats at rest and after exercise.

Publications:

Altland, P. D. and Parker, M. G.: Bilirubinemia and intravascular hemolysis during acclimatization to high altitude. Internat. J. Biometeor. (In press).

REPORTED IN PRESS - 1975

Altland, P. D.: Tolerance of altitude-acclimatized rats to exercise in the cold. Proc. Soc. Exp. Biol. Med. 149: 656-660, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29015-05-LCP

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Digital computer facilities for LCP and LMB

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

PI: William H. Jennings, Jr. Research Physicist A LCP
OTHER: None

COOPERATING UNITS (if any)
Computer Systems Laboratory, DCRT

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Membrane Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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

The laboratory computer facility serving LCP and LMB was in routine operation during 83% of fiscal 1976. Hardware failures accounted for all of the down time. Utilization has been greater than the previous year due to more extensive data processing and this trend will likely continue even though access to the facility is limited.

Project No.: Z01-AM-29015-05-LCP

Project Description:

Program development will continue to emphasize structured data processing programs. This approach using small functional modules permits rapid response to new user needs and easy access for modifications. Documentation of sub-programs and system maintenance will be revised, completing an update of user documentation.

Equipment changes will have to be made in the near future due to decreasing reliability of the existing hardware. To maximize the useful life of the facility, we are working with the Computer Systems Lab of DCRT to partition the data acquisition function onto small, inexpensive dedicated computers. A prototype of the latter will be purchased by July 1, 1976. The results of this project will determine the course of future hardware changes.

Objectives:

Development of a multi-user laboratory computer facility for real-time data acquisition and processing.

Findings:

This facility was in routine operation this year but significant hardware failures necessitated 26 days down time for main frame repairs, 16 days for disc and 12 days for other devices. When operational, utilization has been greater than last year due to more extensive data processing. Program development has proceeded as planned with new modules enhancing the large multi-user programs plus a few special purpose programs. Many older programs were modified to improve usefulness. Documentation of Main programs was brought up to date with the addition of over 80 new writeups and extensive revision of the existing documents.

Significance--This laboratory computer facility is an integral part of certain instrumentation in LCP and LMB. The research projects of a number of investigators in these laboratories incorporate computer based data acquisition and processing as part of the experimental protocol.

Publications:

Shapiro, M. B., Schultz, A. R. and Jennings, W. H.: Computers in the Research Laboratory, Annual Review of Biophysics and Bioengineering, Vol. 5, 177-204, 1976, Palo Alto, Calif.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29016-01-LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Macromolecular assembly reactions

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

PI: James Hofrichter Staff Fellow A LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Spectroscopy and Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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

A light scattering photometer has been built to permit the observation of hemoglobin S aggregation at 1.06 μ. The light scattering properties of concentrated hemoglobin deoxyhemoglobin S solutions have been examined as a function of time after temperature jumps. The scattered intensity can be followed over changes in signal which span over four decades in sensitivity. More complete studies of the light scattering appear warranted and will be pursued to provide information on the mechanism which controls the rate of polymerization. Computer simulations of mechanisms will also be implemented in order to theoretically explore the dynamics of polymer formation. Simple equilibrium and steady-state nucleation models have been shown to provide an adequate description of the concentration, temperature, and solubility dependence of the observed rates, and to rationalize the slowness of the rates observed under the conditions of our experiments.

PHS-6040
(12-75)

Project No: Z01-AM-29016-01-LCP

Project Description:

The project is focussed on the study of the kinetics, thermodynamics, and mechanism of assembly reactions involving macromolecular subunits.

Methods Employed:

Both theoretical and experimental techniques are being used to study assembly reactions. The DC intensity as well as high frequency fluctuations of the light scattered by assembling protein solutions will be studied as a function of angle and concentration in order to obtain information on the molecular weights and virial coefficients of the proteins and non-assembling controls. The intensity of scattered light is also being used as a probe to examine the kinetics of the polymerization and/or assembly reactions. To explain the results of these and our previous experiments, model mechanisms for the nucleation of polymer growth are being developed and computer simulations of these mechanisms will be examined for their ability to accurately describe the observed phenomena. The immediate goal is to provide a detailed model for the nucleation of hemoglobin S polymer growth which is consistent with both the kinetic and thermodynamic results. Relaxation kinetic experiments where birefringence is monitored following small temperature jumps are also being employed as a means of obtaining rate constants for the addition of monomer to polymer.

Major Findings:

The initial results reported here concentrate on the polymerization reaction of hemoglobin S which is responsible for cell sickling and the symptoms of sickle cell disease. From my previous work, it is clear that there is a well defined and reproducible delay before the onset of polymerization in concentrated solutions of deoxyhemoglobin S. The delay is apparent in the reaction as monitored by a variety of independent physical properties, including heat absorption, birefringence, turbidity, viscosity and NMR water proton linewidths and relaxation times. All of these techniques have limited sensitivity and, except perhaps for the water proton linewidths, are incapable of providing direct information on the events which take place during the delay and which determine its length. Intensity light scattering is proportional to the weight average molecular weight. Hence, at constant protein concentration, the formation of dimers would be expected to increase the scattered intensity by a factor of two, with still larger increases expected from more highly aggregated species. Light scattering is thus potentially sensitive to small changes in the distribution of aggregates in the assembling system.

I have modified a conventional light scattering photometer to utilize a 1.06 μ neodymium laster as a light source in order to work in a nearly transparent region of aqueous deoxyhemoglobin solutions. The present instrument is capable of resolving changes in scattered intensity as small as a part in 10^{10} of the incident light. Initial results show that the technique is capable of observing hemoglobin S aggregations at levels at least 100-fold lower than those detectable by the other techniques which have been used to study gelation.

This enhanced sensitivity also allows the polymerization reaction to be monitored at very low degrees of conversion to polymer where the bulk fluidity of the solution is not affected (i.e. at which gelation has not yet taken place). This capability will allow a more complete examination of the early phases of the aggregation reaction and permit the accumulation of physical data on the aggregated molecules while still in solution.

I have also developed a simplified theoretical model for the gelation kinetics. Both steady states rate expressions and an easily understood equilibrium or balanced steady state treatment of the nucleation kinetics have been proposed. To date the time dependence of the progress curves has been examined qualitatively in terms of published nucleation models.

Significance to Biomedical Research and the Program of the Institute:

The kinetics of hemoglobin S gelation provide an attractive and consistent explanation of the known factors which affect the severity of sickle cell disease (see accompanying report # Z01-AM-29009-03-LCP). If the kinetics of gelation do, in fact, control the severity of the disease it is imperative to understand the basic molecular events which are responsible for the delay time. This knowledge may provide new ways of altering the delay and thereby novel approaches to therapy for sickle cell disease.

The basic molecular events involved in the assembly of deoxyhemoglobin S provide a simple and illuminating example of a macromolecular assembly reaction. As such, a fuller understanding of these events will provide a well documented example of the features of such reactions which must be considered in the elucidation of more complex assembly mechanisms.

Proposed Course of Project:

The short term goals of this project have been outlined in the methods section and in the progress report. Light scattering from dilute hemoglobin solutions will be examined to provide both molecular weights and values for the second virial coefficient. These can be compared with known values from osmotic pressure and sedimentation equilibrium experiments. The interpretation of the light scattering experiments on the concentrated solutions which are required for gelation will be complicated by interparticle scattering since the average distance between molecules is only about 30 Å. Consequently the scattered intensity will be studied as a function of concentration up to the gelling concentration (solubility).

The intensity of scattered light will be examined as a function of time for solutions above the gelling concentration. The resulting kinetic curves will be interpreted in terms of weight average degrees of polymerization, relative to that present at the beginning of the reaction. Further, the angular dependence of the scattered intensity will be measured in the resulting polymerized system after reducing the temperature to slow down the kinetics sufficiently to allow serial measurements of the intensity as a function of angle. The resulting data will be integrated with models for the nucleation kinetics which

predict the distribution of aggregate sizes as a function of time in order to obtain a self-consistent mechanism for the kinetics.

In addition, a study of other assembly systems will be initiated in order to select an additional tractable system which will allow the extension of these studies to case which involves the assembly of at least two different protein components.

Publications:

Hofrichter, J., Ross, P. D. and Eaton, W. A.: A thermodynamic and kinetic description of the gelation of deoxyhemoglobin S. Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease, Dallas, Texas, December 1975 (in press).

ANNUAL REPORT
LABORATORY OF PHYSICAL BIOLOGY
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

The Laboratory of Physical Biology is carrying out studies in a broad range of research areas.

Cell Membranes

Lipid complexes. Lipid dispersions in water have provided useful information about the possible physical states and structure of lipids in cell membranes. Thermodynamic analyses of lipid dispersions have been limited by the generally low water solubility of the lipids. This limitation has been circumvented by development of surface tension methods and the use of the Gibbs equation for relating the chemical potentials of the lipid dispersions to the surface tension. These methods have been applied to lecithin dispersion in water to evaluate the relative internal energies of the various condensed states of lecithin in water. The method has also been applied to verify the presence of a lecithin cholesterol 2:1 complex in water which can exist in equilibrium with either an excess of lecithin or an excess of cholesterol.

Studies of the physical properties of the lecithin-cholesterol complex in water have been continued. Low angle X-ray diffraction studies indicate that the complex has a bimolecular spacing of the same magnitude as the gel and liquid-crystal states of lecithin in water. The NMR spectrum of the complex, which was monitored as a function of temperature, indicates that the mobility of the hydrocarbon chain protons is intermediate between the gel and liquid-crystalline states of pure lecithin. Thermodynamic arguments indicate that the stabilizing forces of the complex arise from the association between polar groups in cholesterol and lecithin and that a coplanar array involving interactions between the hydrocarbon chains of lecithin and the fused ring structure of cholesterol is unlikely. (Gershfeld)

Red cell membrane. The hypothesis that cholesterol, the only steroid in mammalian cell membranes, exists in two pools in the human erythrocyte membrane was examined with two new techniques. One study, involving interactions between erythrocyte membranes and the antibiotic pimaricin (which fluoresces in the presence of cholesterol) provided additional support for the two pool hypothesis. The fluorescence occurred in two kinetic stages, and the "average fluorescent lifetime" changed significantly on removal of the cholesterol of one of the presumed pools. A second study examined the cholesterol oxidase catalyzed oxidation of membrane cholesterol. While neither the cholesterol of intact erythrocytes nor the cholesterol of "sealed" ghosts was oxidized, that of "leaky ghosts" and "inside-out" membrane vesicles (which are "sealed") was. In these latter cases the oxidation followed monotonic first-order kinetics for reaction half-times down to 5 minutes and thus did not support the "two-pool" hypotheses. Although this pattern of reactivity of the various membrane preparations suggests that cholesterol is present only at the membrane inner surface, other experimental approaches indicate that at least a major portion is located at the outer surface. The cholesterol oxidase results therefore open doubts on the accepted asym-

metric distribution of phospholipids between inner and outer membrane surfaces, since the evidence for this is based on analogous studies of the reactivities of various membrane preparations with phospholipases. (Gottlieb)

Muscle Contraction

X-ray diffraction in different physiological states. Equatorial X-ray diffraction patterns from resting and activated sartorius muscles were obtained using an electronic position sensitive detector with 100 μm resolution. Electronic gating was designed to record patterns from different physiological states: (1) resting state before activation, (2) isometric contraction at long sarcomere length, (3) isotonic contraction, (4) isometric contraction at short sarcomere length. The load of shortening ranged between 0.14 and 0.40 times the steady isometric value. While the ratio of the intensities I_{10}/I_{11} decreased approximately by a factor of five upon activation, it remained essentially the same for both isometric and isotonic contraction. Analysis of the 10 and 11 reflections separately showed that I_{10} and I_{11} both increased by a small amount in the transition from the isometric to isotonic state. The results show that once the muscle is fully activated, changes in force have much smaller effect on the equatorial reflections than the original transition from rest to activation. The data provide evidence that the drop in tension in a shortening muscle is due primarily to changes in the force and configuration of the individual cross-bridges rather than the total number. (Podolsky, St. Onge, Yu, Lymn)

Interpretation of the diffraction patterns. The equatorial reflections from muscle provide information in only two dimensions. Until now, only the two principal equatorial reflections have been studied in detail, and they have been interpreted in terms of radial movements by projections from the thick myosin-containing filaments. However, there are other reflections, which are particularly visible using the new camera-counter system, and a study was undertaken to discover how much information could be obtained from the additional data.

A computer program was developed that accepts a three dimensional model of actin and myosin packing and computes expected intensities for equatorial reflections and axial layer lines. It is possible, using this program, to move the myosin cross-arms in a manner thought to occur in activated muscle, and observe the changes in the predicted X-ray pattern. This work confirms that, if the thick filament is either three or four stranded, then the myosin cross-arms in a relaxed muscle reach out to the radius of the actin filament. Azimuthal change that does not disturb the helical nature of the myosin cross-arms will not cause a change in the relative intensities of the low order equatorial reflections. Thus the change of the ratio of intensities of the first two reflections seen upon activation does arise from actin-myosin cross-linking. The amount of change seen is also dependent on the configuration of the linkage. (Lymn)

Synchrotron radiation. Equatorial diffraction patterns from resting sartorius were obtained using the synchrotron radiation source SPEAR at Stanford Linear Accelerator Center, recorded by the position sensitive detector. Under comparable conditions, it is estimated that the increase in intensity is between 25 and 50 times to that obtained on a 0.8 kW GX-6 Elliott rotating anode generator. (Yu, Lymn)

Mechanical experiments. The method developed previously, for determining stiffness by measuring the propagation speed of small mechanical disturbances, was studied further. This method has the distinct advantage of having a very small "measurement time," on the order of 15 μsec, so that the state of the muscle does not change during the course of the measurement. The method was further substantiated by demonstrating that during isometric tension development at different muscle lengths (different amounts of overlap between thick and thin filaments) the increment in measured stiffness upon activation was roughly proportional to cross-bridge number.

The technique was also used to determine the time course of stiffness and force development following initiation of a twitch. It was found that stiffness led force by about 25 msec at 6°C and 4 msec at 26°C. Once the influence of the tendon is separated from the muscle sarcomere behavior, this finding will provide valuable information about the mechanism of cross bridge force development. To this end, a device has been developed which makes it possible to monitor the sarcomere movement directly and non-invasively using laser diffraction techniques. (Schoenberg)

The technique of puncturing the mammalian muscle cell membranes with mild glycerol treatment, allowing direct activation of the fibers with applied calcium solutions, has been applied to fibers from two different types of muscles of the guinea pig. The isotonic contraction properties of fiber bundles from a slow (soleus) and a fast (extensor digitorum longus) muscle were compared under the same chemical conditions. The results show unequivocally that the differences in the contractile properties of the slow and the fast muscles represent fundamental differences in their cross bridge kinetics rather than differences in intracellular milieu. (Gulati)

The activation mechanism. The new method for measuring isometric force and ^{45}Ca loss continuously in segments of single skinned muscle fibers has been extended and applied to studies of unstimulated and stimulated intracellular ^{45}Ca movements in situ. Unstimulated ^{45}Ca loss is very slow relative to stimulated loss. Separation of net loss into efflux and influx components, by EGTA chelation of ^{45}Ca that has left the sarcoplasmic reticulum, shows that most of the resting efflux is reaccumulated by the active influx system, but that efflux proper is slow. High free Mg ion, which strongly inhibits net Ca release, has little effect on resting efflux. Caffeine, in low Mg ion solutions, stimulates efflux proper; this result confirms, in situ, a mechanism of caffeine action that has been in question.

Strong "depolarization," produced by application of a large Cl gradient across the internal membranes, causes a large transient increase in ^{45}Ca efflux at moderate free Mg ion level. The presence of EGTA inhibits the response nearly completely, showing for the first time that free Ca is required for "depolarization"-induced release. (Stephenson)

Electrical properties of heart muscle. Previous work on the passive properties of sheep cardiac Purkinje fibers has been extended to look at the influence of active currents, particularly those that might be generated by the membrane between adjacent cells in the body of the fiber. It was found that potassium and calcium currents across this membrane mirror the currents on the surface (to the extent that the membranes are similar). However, it was demonstrated that any inward sodium current generated by this membrane does not appear at the surface and therefore is unlikely to be important in excitation. (Schoenberg)

Morphogenesis

Cellular organelles. Previous studies of Euglena gracilis led to the conclusion that all macromolecules were bound in vivo to large organelles. The arrangement of enzyme is probably not a random attachment, but rather a spatial pattern related to the biochemical sequence of reactions that they catalyze. Upon disruption of cells, there is a release of those enzymes which were weakly bound, while others remain attached to mitochondria, lysosomes and other "particulates." The use of bifunctional reagents now offers the possibility of determining both the association of an entire pathway with a specific organelle and also the spatial arrangement of the individual enzymes of a biochemical sequence.

The synthesis of the amino acid tryptophan is accomplished via twelve enzymatic steps starting with erythrose-4-phosphate; the same pathway is found throughout the bacteria, algae and fungi. The first seven steps, up to chorismic acid, are common to other pathways which lead to phenylalanine, tyrosine, para-aminobenzoic acid and the isoprenoid quinones. In Euglena gracilis, this 12-step pathway is more highly organized than in any other species--or at least can be extracted in more highly organized units. We have found that the first enzyme sediments with large cell particulates. Enzymes 2 through 6 are contained in a small complex of $1\text{-}2 \times 10^5$ MW. Enzyme 7 and 8 are extracted "unattached" (although reaction #8 is probably several biochemical steps and therefore may be a "complex" of enzymes). Enzymes 9 through 12 are joined in a single unit of 234,000 MW. These complexes are extractable and stable; it is possible that in vivo they may be even further co-joined. No other organism has been found to have such a high degree of organization in this pathway. It is even more remarkable that so many enzymatic steps should be contained within so small a set of molecular weights. (Kempner)

Cellular growth. When cultures of Euglena gracilis in balanced growth are transferred from a minimal to a complex medium, the mean cell volume changes with little or no change in specific growth rate. This is in contrast to the widely-accepted finding that in bacteria there is a simple relationship between cell size and the growth-supporting ability of the chemical environment. It seems possible that during cultivation in complex media, Euglena might have different regulatory mechanisms than those of the enteric bacteria. Environmentally induced variation in cell volume and composition are providing useful experimental approaches for studying growth and division of Euglena. (Shehata, Kempner)

Vitamin B_{12}. Vitamin B_{12} starvation of Euglena is accompanied by cell enlargement and a gradual decrease in the specific growth rate. Vitamin B_{12} deficiency appears to bring about the arrest of cell division of Euglena gracilis. Experimentally induced B_{12} starvation proved to be a useful technique to generate synchronous growth of Euglena. An important method of evaluating the synchronous growth of Euglena was provided by following the distribution of cell volume during the division cycle. A sequence of such distributions was obtained during synchronous growth of Euglena gracilis. During division, one peak is formed by newly-born cells, which translates and spreads as cells grow, while another peak is formed by cells which have not yet divided. (Shehata, Kempner)

Insect metamorphosis. Insect metamorphosis is a programmed process under hormonal control which entails the decay and destruction of the larval cells concomitantly with the differentiation of the cells and structures that will give rise to the adult insect. The greater part of this process has taken place within the span of a week. This telescoping of the aging and development process in the insect might be expected to bring forth features otherwise unobservable in the corresponding processes of man and mammals, which take place in a much more expanded time scale. In particular, the study of the fate of proteins, ribosomes and ribosomal RNA refers to the fate of essential components of the protein synthesizing machinery and its products during aging and development.

Improved and novel methods, involving various chromatographic procedures and isoelectric focusing, have been used to purify and isolate calliphorin. "Calliphorin" as previously prepared has now been shown to be a mixture of two, and maybe three, closely associated proteins. The blood titer of calliphorin has been measured; its level increases by almost two orders of magnitude during larval growth, and then abruptly declines some two days before pupariation. (Levenbook) Conditions for maintaining isolated larval fat body in organ culture have been perfected. Such cultures release a variety of proteins into the medium, including radioactive proteins when the medium is supplemented with tagged amino acids. This technique is being used to prepare labeled calliphorin, which is then isolated from pooled medium. Simplified procedures for calliphorin isolation from such media are presently under study. (Pau) To try and explain the dramatic

switch-off of calliphorin biosynthesis, the effects of molting hormone (β-ecdysone) has been examined. In the presence of physiological concentrations of β-ecdysone, substantially less ^{35}S-methionine is incorporated into calliphorin as measured by immunoassay. Heretofore, β-ecdysone has been thought to increase the rate of protein synthesis. (Bauer, Pau)

The calculated rates of rRNA biosynthesis during fly metamorphosis have been refined by more accurately estimating short-term incorporation of tagged precursor into true rRNA as opposed to rRNA contaminated with heterogeneous RNA and by the use of an iterative computer program (MLAB) for the long-term labeling decay measurements. The revised data from the two types of experiments are in good agreement, and now show that almost the entire ribosomal pool is replaced during metamorphosis of the fly. (Protzel, Levenbook)

Mature larvae of many insects accumulate so-called storage granules in the fat body. Such granules in the blowfly C. vicina differ widely in density, but are otherwise apparently similar. They are virtually free of uric acid, are insoluble in aqueous solvents in the absence of denaturing agents such as SDS or urea, are composed of over two dozen proteins among which calliphorin predominates. (Levenbook)

The definitive and unique structure of the paragonial substance from male D. melanogaster has now been elucidated. The compound is 4(1-β-galactopyranosyl-X-glycerol) phosphorylethanolamine. (Chen, Fales)

Human red cell sickling. Sickle cell disease is a non-infectious hereditary anemia in which the disease process is well understood at the molecular level; the defective molecule has not only been identified as an abnormal hemoglobin (Hb S), but the abnormal interactions of this molecule can be explained. Observations reported from many laboratories are consistent with an aggregation mechanism due to hydrophobic interactions by deoxy Hb S. Work on Hb S has advanced both in vitro and in vivo. Both aggregation and solubility of Hb S are inhibited by inorganic ions in the order of the classical Hofmeister series. Red cells appear to increase in volume upon "sickling." This change, opposite in direction to published claims or predictions, is in the direction of the volume increase during sickling observed by Murayama in his high hydrostatic pressure experiment and with his thermodynamic prediction of the volume of activation. This increase cannot come from "organized" water because sickling is an entropy driven process--organized water appears to be "boiled off" from between the adjacent deoxy Hb S; some increase in volume comes from the tubular space created in the center of hollow cables (the six stranded microtubules). (Murayama, Levine, Shehata)

In collaboration with Dr. Oku Ampofo, Dr. J. Obeng, and Dr. M. Appiah in Ghana, additional data have been obtained supporting the effectiveness of oral carbamide (urea) in prevention of crisis and ameliorating the symptoms of sickle cell crises. (Murayama)

Biological Rhythms

Firefly flash synchronization. The adaptive significance of firefly flash synchronization and evolution of this unique communal behavior is not entirely clear. The basic problem is that since synchronization is a group behavior it cannot in itself promote the reproductive fortunes of the individual male, which it must do, according to Darwinian natural selection theory, if the behavior is to be perpetuated genetically. A possible solution to this problem is that sexual selection by the female is on the basis of male flash intensity, which requires simultaneity of presentation because of immediate refractoriness in the response system; the simultaneity requirement in turn provides built-in protection against out-of-phase mutant "cheaters," making the synchronous behavior a true, and rare, example of group adaptation. (J. Buck, E. Buck)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 27000-14 LPB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
The Mechanism of Muscular Contraction

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

PI:	R. J. Podolsky	Chief, LPB	LPB NIAMDD
OTHER:	R. W. Lymn	Senior Staff Fellow	LPB NIAMDD
	L. C. Yu	Senior Staff Fellow	LPB NIAMDD
	E. W. Stephenson	Guest Worker	LPB NIAMDD
	J. Gulati	Staff Fellow	LPB NIAMDD
	J. Hartt	Staff Fellow	LPB NIAMDD

COOPERATING UNITS (If any)
None

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Cellular Physics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.5	5.5	2

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

Muscle cells are activated when calcium is released from the sarcoplasmic reticulum into the myofilament space. The processes involved in this intracellular calcium movement will be studied by measuring isometric force and ^{45}Ca fluxes in mechanically skinned fibers from frog muscle. The structural changes associated with the transition of muscle cells from one physiological state to another will be examined by X-ray diffraction techniques, using radiation from conventional rotating anode generators and synchrotrons in conjunction with electronic, position sensitive, X-ray detectors. The rate constants associated with the turnover of actomyosin cross-bridges will be studied by analyzing the contraction kinetics of skinned muscle fibers under controlled chemical conditions.

PHS-6040
(12-75)

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. X-ray diffraction patterns from resting and electrically activated muscle cells were examined with a high resolution, position sensitive, electronic detector. Upon activation, the intensities of the principal reflections, 10 and 11, change reciprocally. A newly derived form factor that accepts a three-dimensional model of actin and myosin packing was used to show that these intensity changes are probably caused by attachment of the myosin projections to actin rather than by radial or azimuthal movement of the myosin projections *per se*.

2. The ratio of the intensities of the principal reflections in activated muscle is nearly the same in shortening and non-shortening preparations. This indicates that the drop in tension in shortening muscle is due primarily to a change in the force and configuration of individual cross-bridges rather than to a change in the number of cross-bridges.

3. Equatorial diffraction patterns from resting muscle were obtained using synchrotron radiation. This source reduced the time required to obtain an X-ray diffraction pattern by about 50 fold, which makes it possible to study the structural changes associated with both transient and steady muscle motions.

4. The technique of puncturing the cell membranes of mammalian muscle cells with mild glycerol treatment was applied to fiber bundles from a slow (soleus) and a fast (extensor digitorum longus) muscle of the guinea pig. Differences in the contractile properties of the slow and fast muscles were found to be due to differences in the kinetics of cross-bridge turnover in the two fiber types rather than to differences in intracellular milieu.

5. Intracellular calcium movements in isolated skinned muscle fibers were examined with an improved tracer method. Caffeine directly stimulates calcium efflux from the sarcoplasmic reticulum of skinned muscle fibers. A strong depolarization also induces net calcium release from the sarcoplasmic reticulum in the presence of 10^{-4} M free magnesium. The depolarization response is calcium dependent since it is strongly inhibited by chelation of the released calcium.

Significance to Biomedical Research and the Program of the Institute:

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 effect of motion on the number of cross bridges in intact, electrically-stimulated muscle fibers will be studied by X-ray diffraction.
4. Movement of calcium and magnesium between the myofilament space and the sarcoplasmic reticulum will be studied by tracer methods.

Publications:

Podolsky, R. J., St. Onge, R., Yu, L., and Lymn, R. W.: X-ray diffraction of actively shortening muscle. Proc. Nat. Acad. Sci. USA 73: 813-817, 1976.

Podolsky, Richard J.: The Kinetics of Cross-Bridge Turnover. In Heilmeyer, L.M.G., Ruegg, J. C., and Wieland, Th. (Eds.): Molecular Basis of Motility. Heidelberg, Springer-Verlag, 1976, pp. 53-68.

Lymn, Richard W., and Cohen, Gerson H.: Equatorial X-ray reflections and cross arm movement in skeletal muscle. Nature 258: 770-772, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 27001-02 LPB

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Contractility and Excitability of Skeletal and Cardiac Muscle

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

PI: Mark Schoenberg Medical Officer LPB NIAMDD

COOPERATING UNITS (if any)

Dr. Jay B. Wells, Medical Neurology Branch, NINCDS

LAB/BRANCH

Laboratory of Physical Biology

SECTION

Section on Cellular Physics

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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

In the coming year, work will proceed along two main lines. The study to determine the mechanism of muscle contraction will concentrate upon separating sarcomere motion from motion at the ends of the muscle, the former being greatly influenced by tendon compliance. In studying this we will make use of optical diffraction techniques. Stiffness measurements based upon the speed of propagation of small mechanical disturbances, will be used as a measure of cross-bridge interaction during isotonic shortening.

Work on the study of excitability in cardiac Purkinje fibers will be directed towards illucidating the effect of ionic currents generated in clefts of the fiber upon excitation.

PHS-6040
(12-75)

Project Description:

Goals:

1) To understand the molecular and structural basis of muscular contraction.
2) To determine the factors controlling the excitability of heart muscle.

Methods and Major Findings, Goal 1

1) The method developed previously, for determining stiffness by measuring the propagation speed of small mechanical disturbances, was studied further. This method has the distinct advantage of having a very small "measurement time," on the order of 15 μsec, so that the state of the muscle does not change during the course of the measurement.

2) The method was further substantiated by demonstrating that during isometric tension development at different muscle lengths (different amounts of overlap between thick and thin filaments) the increment in measured stiffness upon activation was roughly proportional to cross-bridge number.

3) The technique was used to determine the time course of stiffness and force development following initiation of a twitch. It was found that stiffness led force by about 25 msec at 6°C and 4 msec at 26°C.

4) Once the influence of the tendon is separated from the muscle sarcomere behavior, finding 3) will provide valuable information about the mechanism of cross bridge force development. To this end, a device has been developed which enables us to monitor the sarcomere movement directly and non-invasively using laser diffraction techniques.

Methods and Major Findings, Goal 2

1) In FY 74 we studied some of the passive properties of sheep cardiac Purkinje fibers. In pursuing goal 2, we extended this analysis last year to include active properties of these fibers.

2) To the extent that the cell membranes distributed through the volume of the Purkinje fiber (the "off surface" membrane) is active, the potassium and Ca Ca across this membrane mirror, to first order, the potassium and calcium currents on the surface.

3) In contrast, inward sodium current generated across these "off surface" membranes seems to have little relationship to currents generated on the surface. As such it is useful only for discharging the extra capacity of the "off-surface" membrane and does not contribute much to the excitation of the surface membrane.

Significance to Biomedical Research and the Program of the Institute:

The experiments on the mechanism of muscular contraction are significant for two reasons. First they contribute to a sound understanding of how normal muscle functions (which is vital to any study of diseased muscle) and secondly, the same type of structure responsible for muscle contraction appears to be common to many other cells in which motility plays a role such as platelets, nerve axons, and many others.

The study of heart excitability is of course of great interest in that a fairly large fraction of deaths from myocardial infarction are related not to the physical destruction of large amounts of tissue, but rather to small injuries which result in destruction of normal electrical activity. One question our work has shed light on is the question of how the geometry of Purkinje fibers might be important in coordinating electrical activity. Another is what factors are necessary to describe the state of excitability of heart muscle (and other cells as well) and also how might these factors be influenced by other parameters in the system such as potassium concentration, drugs, etc.

Future Course

Goal 1

1) Again, differentiating between effects of compliance in the tendon and that in the sarcomere continues to have high priority. We plan to use the device we have developed for measuring sarcomere movement as part of a feedback loop in order to control the sarcomere movement, irrespective of the tendons.

2) We will use our techniques for measuring cross bridge interaction (stiffness) under different physiological conditions which will tell us about the rate constants for making and breaking bridges.

Goal 2

1) We intend to examine how "off surface" membrane effects excitability.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-27002-13

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Sense of rhythm

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

PI: John B. Buck — Research Physiologist — LPB NIAMDD

COOPERATING UNITS (if any) 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; Dr. Jean-Marie Bassot, Laboratory de Bioluminescence, Centre Nationale de Recherche Scientifique, France.

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Comparative Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	1	2

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

Our diversion from the mechanism of firefly flash synchronization to the adaptive significance and evolution of this unique communal behavior, mentioned last year, proved sufficiently engrossing to lead to a full-dress theoretical analysis in a paper now in press. The basic problem is that since synchronization is a group behavior it cannot in itself promote the reproductive fortunes of the individual male, which it must do, according to Darwinian natural selection theory, if the behavior is to be perpetuated genetically. Our suggested solution is that sexual selection by the female is on the basis of male flash intensity, which requires simultaneity of presentation because of immediate refractoriness in the response system; the simultaneity requirement in turn provides built-in protection against out-of-phase mutant "cheaters," making the synchronous behavior a true, and rare, example of group adaptation.

PHS-6040
(12-75)

Project Description:

Objectives:

1. Our diversion from the mechanism of firefly flash synchronization to the adaptive significance and evolution of this unique communal behavior, mentioned last year, proved sufficiently engrossing to lead to a full-dress theoretical analysis in a paper now in press. The basic problem is that since synchronization is a group behavior it cannot in itself promote the reproductive fortunes of the individual male, which it must do, according to Darwinian natural selection theory, if the behavior is to be perpetuated genetically. Our suggested solution is that sexual selection by the female is on the basis of male flash intensity, which requires simultaneity of presentation because of immediate refractoriness in the response system; the simultaneity requirement in turn provides built-in protection against out-of-phase mutant "cheaters," making the synchronous behavior a true, and rare, example of group adaptation. (J. Buck; E. Buck).

2. The paper describing the ultrastructure and certain biochemical properties of firefly photocyte granules was completed and submitted for publication. We conclude that these particulates are peroxisomes. (C. Hanna, T. Hopkins, J. Buck).

3. In the final stages of our analysis of firefly pacemaker physiology we discovered a long series comprising randomized single external signal intrusions on Pteroptyx cribellata, which complements ideally our prior records of steady state driving and confirms the concept of cycle-by-cycle resetting of the flash-control oscillator. (J. Buck; E. Buck).

Significance to NIAMDD Research:

The new data on rhythms and entrainment encourage the hope of eventually defining human pacemaking activity, separating timing from motor steps and understanding the ability to entrain to external pacing.

Proposed Course of Project:

No major changes anticipated.

Publications:

Bassot, Jean-Marie: Les cellules lumineuses du coleoptere Phengodes. Recherches Biol. Contemporaines 1: 79-96, 1974.

Bassot, J. M.: Les organes lumineux à Bactéries symbiotiques de quelques téléostéens leiognathides. Arch. de Zool. Exp. & Gen. 116: 359-373, 1975.

Hanna, C. H., Hopkins, T. A. and Buck, J.: Peroxisomes of the firefly lantern. J. Ultrastructure Res. (In Press).

Buck, J. and Buck, E.: Synchronous fireflies. Scientific Amer.: 234: 74-85, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 27003-07 LPB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
The Dynamic Properties of Cell Membranes and Related Systems

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

PI:	Norman L. Gershfeld	Research Chemist	LPB NIAMDD
OTHER:	Ann E. Kaplan	Chemist (Biochem.)	NCI

COOPERATING UNITS (if any) S. Rasin, Department of Clinical Microbiology, Hadassah Medical School, Jerusalem, Israel

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Laboratory of Physical Biology

SECTION
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INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.75	1.75	1

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

Lipid dispersions in water have provided useful information about the possible physical states and structure of these lipids in cell membranes. Thermodynamic analyses of these systems have been limited by the generally low water solubility of the lipids. This limitation has been circumvented by development of surface tension methods and the use of the Gibbs equation for relating the chemical potentials of the lipid dispersions to the surface tension. These methods have been applied to lecithin dispersions in water to evaluate the relative internal energies of the various condensed states of lecithin in water. The method has also been applied to verify the presence of a lecithin/cholesterol 2:1 complex in water which can exist in equilibrium with either an excess of lecithin or an excess of cholesterol.

Formation of the lecithin-cholesterol complex in water was reported last year, and these studies have been continued to further characterize the physical properties of the complex. The NMR spectrum of the complex was monitored as a function of temperature and indicates that the mobility of the hydrocarbon chain protons is intermediate between the gel and liquid-crystalline states of pure lecithin.

PHS-6040
(12-75)

Project Description:

The principal objective of this project is to establish a physical-chemical basis for understanding the dynamic properties of cell membranes.

Major Findings:

I. Physical-chemical properties of membrane lipids (Gershfeld)

A. Thermodynamics of lipid aggregates in water

Lipid dispersions in water have provided useful information about the possible structural arrangement of these lipids in cell membranes. However, the generally poor water solubility of these lipids has limited the measurement of thermodynamic properties of the aqueous dispersions. These limitations have been circumvented by utilizing the fact that even the most poorly soluble of the lipids will still lower the surface tension of water. The Gibbs equation relates the chemical potentials of lipids in suspension with the surface tension of the aqueous solution. Two examples may be cited where the method has been applied: (a) The gel $\rightleftarrows$ liquid-crystal transition for dimyristoyl lecithin (DML) suspensions in water occurs at 23.5°C accompanied by a latent heat λ_c. From the temperature dependence of the surface tension of the DML dispersions, λ_c and the transition temperature may also be obtained; the values for DML were in agreement with direct calorimetric measurements of the temperature and heat of transition. (b) Studies with lecithin-cholesterol mixtures, utilizing largely non-thermodynamic methods have reported complexes with a variety of stoichiometries. Surface tension studies of lecithin-cholesterol mixtures indicate that when the molar ratio lecithin/cholesterol is greater than 2:1 two lipid phases exist--one is pure lecithin and the other a complex of lecithin and cholesterol (molar ratio 2:1). When the lecithin/ cholesterol ratio is less than 2:1, excess cholesterol forms a separate phase in equilibrium with the lecithin-cholesterol (2:1) complex. The complex has been isolated by isopycnic centrifugation. These results have been duplicated with other membrane phospholipids.

B. NMR spectroscopy of lipids

Continuous wave NMR studies of anhydrous mixtures of lecithin and cholesterol at various temperatures and composition indicate that for the protons of the aliphatic moiety in lecithin, characteristic changes in proton mobility occur. These changes follow predictably the phase diagram for anhydrous mixtures of lecithin and cholesterol reported last year. These NMR observations are similar to those reported for aqueous suspensions of lecithin-cholesterol mixtures and therefore indicates that the anhydrous structures are retained in the presence of water.

II. Dynamics of membrane formation in mycoplasma (Kaplan)

Techniques for growing the mycoplasma A. laidlawii have been set up in this laboratory. These organisms were selected because (a) they are dependent upon exogenous lipids for growth, incorporating directly into their plasma membranes the exogenous fatty acids, and (b) the plasma membrane is the only identifiable membrane in the organism. Thus it will be possible to study the dynamics of membrane phospholipid synthesis by using radiotracers of lipids in a defined growth medium.

Future Course:

(a) X-ray diffraction studies of the structure of the lecithin-cholesterol complex are now in progress. (b) Thermodynamic studies of lipid suspensions in water using the surface tension approach will be continued. Of particular interest will be the search for a discrete bilayer lecithin phase which can form spontaneously. (c) The dynamics of fatty acid incorporation into mycoplasma membranes will be studied using a variety of fatty acids and experimental conditions.

Publications:

Tajima, Kazuo and Gershfeld, N. L.: Latent heat of transition between crystalline polymorphic states of cholesterol by equilibrium spreading pressures. J. Colloid Interface Sci. 52: 619-620, 1975.

Gershfeld, Norman L.: Physical chemistry of lipid films at fluid interfaces. Ann. Rev. Phys. Chem., in press.

Gershfeld, N. L. and Tajima, K.: Energetics of the transition between lecithin monolayers and bilayers. J. Colloid Interface Sci., in press.

Reported in Press, 1975

Tajima, K. and Gershfeld, N. L.: Thermodynamics of monolayer solutions of lecithin and cholesterol mixtures by the surface vapor pressure method. Adv. in Chem. Series, No. 144, 165-176, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 27004-06 LPB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Physical Chemistry

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

PI: Melvin H. Gottlieb Research Chemist LPB NIAMDD

COOPERATING UNITS (if any)

Dr. R. Chen, IRTD/NHLI

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Comparative Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.67	1	0.67

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

Work of this past year tested an earlier hypothesis that cholesterol, the only steroid in mammalian cell membranes, exists in two pools in the human erythrocyte membrane. This hypothesis was supported by one study involving interactions between erythrocyte membranes and the antibiotic pimaricin, which fluoresces in the presence of cholesterol. The fluorescence occurs in two kinetic stages, and the "average fluorescent lifetime" changed significantly on removal of the cholesterol of one of the presumed pools. A second study examined the cholesterol oxidase catalyzed oxidation of membrane cholesterol. While neither the cholesterol of intact erythrocytes nor the cholesterol of "sealed" ghosts was oxidized, that of "leaky ghosts" and "inside-out membrane vesicles (which are "sealed") was. In these latter cases the oxidation followed monotonic first-order kinetics for reaction half-times down to 5 minutes and thus did not support the "two-pool" hypotheses. Although this pattern of reactivity of the various membrane preparations seemingly indicates that cholesterol is present only at the membrane inner surface, there is independent evidence that at least a major portion is located at the outer surface. The cholesterol oxidase results therefore open doubts on the accepted asymmetric distribution of phospholipids between inner and outer membrane surfaces, which is based on analogous studies of the reactivities of various membrane preparations with phospholipases.

Project Description:

Studies of the past year continued previous work on the state of cholesterol in erythrocyte membranes. This earlier work, which involved extraction with plasma lipoproteins, had suggested that cholesterol exists in two states in the membrane, 35% readily extractable and the remainder firmly bound. The responses of membrane cholesterol to two other reactions were examined for further evidence of two states. The first reaction was with the antibiotic pimaricin, which fluoresces in the presence of cholesterol. Rapid reaction kinetic measurements showed that the fluorescence developed in two well defined stages, one with a time constant of less than 10 msec and the other with a time constant of about a second. The results were not, however, completely consistent with the extraction experiments, since the fluorescent intensities of the two stages, which are presumably proportional to the quantities of cholesterol involved, are in a 50:50 ratio, rather than the 35:65 ratio predicted by the extraction experiments. Also consistent with, but not proving, the two-state hypothesis was the finding that the "average fluorescent lifetime" was significantly changed by removing the extractable cholesterol. The second reaction of membrane cholesterol studied was its cholesterol oxidase catalyzed oxidation to form Δ4- cholestenone and H_2O_2. The cholesterol of intact erythrocytes was not oxidized in the presence of this enzyme, but that of "leaky" (see below) ghosts was. The reaction followed monotonic first order kinetics for reaction half-times down to 5 minutes and thus did not support the hypothesis of two cholesterol pools. The pattern of reactivity of the various membrane preparations is of significance: the cholesterol of intact erythrocytes, and of "resealed" ghosts when the enzyme was present only outside the ghosts, was not oxidized while the cholesterol of leaky ghosts, ghosts "resealed" with the enzyme inside, and "inside-out" membrane vesicles, which are "sealed," was oxidized. Thus the pattern of reactivity of the various preparations would indicate that cholesterol is present only on the inner surface of the membrane. However, there is independent evidence (the earlier mentioned extraction experiments, exchange and freeze fracture studies of others) that at least a major portion of the cholesterol is at the outer membrane surface. It therefore follows that location on a side of the membrane accessible to the enzyme is not sufficient for reactivity with the enzyme. The important consequence of this is that it casts doubt on the accepted asymmetric distribution of the various phospholipids between inner and outer membrane surfaces, which are based on analogous studies of the pattern of reactivities of membrane preparations with phospholipases. Since the enzymatic reaction of even the inner surface membrane cholesterol is 200 X slower than in solution, a detailed study of its mechanism is planned.

Publications:

Gottlieb, M. H.: The limited depletion of cholesterol from erythrocyte membranes. Biochim. Biophys. Acta 433: 333-343 (1976).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-27006-19

PERIOD COVERED: July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Developmental biochemistry of insect metamorphosis

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

PI:	L. Levenbook	Research Chemist	LPB NIAMDD
OTHER:	A. Bauer	Research Physiologist	LPB NIAMDD
	A. Protzel	Visiting Fellow	LPB NIAMDD
	R. Pau	Visiting Scientist	LPB NIAMDD

COOPERATING UNITS (if any)

Dr. H. Fales Z01 HL-01003-05

LAB/BRANCH

Laboratory of Physical Biology

SECTION

Comparative Physiology Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

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

(1) All so-called "pure" preparations of the blowfly protein calliphorin, both from this and other laboratories, are composites of two or three closely similar proteins. (2) Blowfly fat body in organ culture liberates labeled proteins, including calliphorin, into the medium. Labeled calliphorin is being prepared from this source. (3) The *in vitro* synthesis of calliphorin appears to be inhibited by the molting hormone ecdysone. (4) The rates of ribosomal RNA degradation and synthesis during fly metamorphosis have been revised. Ribosomes of the adult fly are almost entirely made *de novo*. (5) Granular hemocytes (phagocytes) have been isolated and maintained in cell culture. These cells *in vitro* engulf a variety of foreign particles. (6) A unique natural compound isolated from the male accessory glands of *Drosophila melanogaster* has been characterized as 4(1-β-galactopyranosyl-X-glycerol) phosphoethanolamine.

PHS-6040
(12-75)

Project Description:

Objectives:

1. Elucidation of the structure and function of the insect protein calliphorin.

2. Verification of previously measured ribosomal turnover rates during fly metamorphosis with the aid of a computer program.

3. Isolation, in vitro culture and biochemical characterization of fly phagocytic blood cells during metamorphosis.

4. A description of the chemical nature, origin and fate of fat body storage granules in blowfly larvae.

5. Elucidation of chemical structure of male Drosophila accessory gland substance.

Methods Employed:

Routine physical, chemical and immunological methods for calliphorin and nucleic acids; computer analysis of ribosomal kinetics; NMR and mass-spectroscopy; in vitro cell and organ culture.

Major Findings:

1. Improved and novel methods, involving various chromatographic procedures and isoelectric focussing, have been used to purify and isolate calliphorin. "Calliphorin" as previously prepared both by ourselves and others, has now been shown to be a mixture of two, and maybe three, closely associated proteins. Characterization of "chemically pure" calliphorin remains to be determined.

2. The blood titer of "calliphorin" has been measured; its level increases by almost two orders of magnitude during larval growth, and then abruptly declines some two days before pupariation.

3. Conditions for maintaining isolated larval fat body in organ culture have been perfected. Such cultures release a variety of proteins into the medium, including radioactive proteins when the medium is supplemented with tagged amino acids. This technique is being used to prepare labeled "calliphorin," which is then isolated from pooled medium. Simplified procedures for calliphorin isolation from such media are presently under study.

4. To try and explain the dramatic switch-off of calliphorin biosynthesis [see (2) above], the effects of molting hormone (β-ecdysone) has been examined. In the presence of physiological concentrations of β-ecdysone, substantially less ^{35}S-methionine

is incorporated into "calliphorin" as measured by immunoassay. Heretofore, β-ecdysone has been thought to increase the rate of protein synthesis.

5. The calculated rates of rRNA biosynthesis during fly metamorphosis have been refined by more accurately estimating short-term incorporation of tagged precursor into true rRNA as opposed to rRNA contaminated with heterogeneous RNA and by the use of an iterative computer program (MLAB) for the long-term labeling decay measurements. The revised data from the two types of experiments are in good agreement, and now show that almost the entire ribosomal pool is replaced during metamorphosis of the fly.

6. Mature larvae of many insects accumulate so-called storage granules in the fat body. Such granules in the blowfly C. vicina differ widely in density, but are otherwise apparently similar. They are virtually free of uric acid, are insoluble in aqueous solvents in the absence of denaturing agents such as SDS or urea, are composed of over two dozen proteins among which calliphorin predominates.

7. Procedures for the isolation, and the optimum medium for their in vitro culture, have been worked out for C. vicina phagocytic blood cells (granular hemocytes). The cytology of these cells have been examined in the E.M., and their phagocytic abilities in vitro monitored by the uptake of polystyrene beads.

8. The definitive and unique structure of the paragonial substance from male D. melanogaster has now been elucidated. The compound is 4(1-β-galactopyranosyl-X-glycerol) phosphorylethanolamine.

Significance to NIAMDD Research:

Insect metamorphosis is a programmed process under hormonal control which entails the decay and destruction of the larval cells concomitantly with the differentiation of the cells and structures that will give rise to the adult insect. The greater part of this process has taken place within the span of a week. This telescoping of the aging and development process in the insect might be expected to bring forth features otherwise unobservable in the corresponding processes of man and mammals, which take place in a much more expanded time scale. In particular, the study of the fate of proteins, ribosomes and ribosomal RNA refers to the fate of essential components of the protein synthesizing machinery and its products during aging and development.

Proposed Course of Projects:

Continuation of studies on structure and function of both calliphorin and of storage granules. Further investigation of factors involved in

tissue phagocytosis by hemocytes during metamorphosis.

Publications:

Levenbook, L., Sridhara, S. and Lambertsson, A.: Extracellular ribosomes during metamorphosis of the blowfly Calliphora vicina -- a reappraisal of their authenticity. Biochem. Biophys. Res. Comm. 70: 15-21, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM-27007-14

PERIOD COVERED
July 1, 1975 through June 30, 1976.

TITLE OF PROJECT (80 characters or less)

Human red cell "sickling" and "desickling"

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

PI:	M. Murayama	Research Chemist	LPB NIAMDD
OTHER:	A. Levine	Staff Fellow	LPB NIAMDD

COOPERATING UNITS (if any)

Dr. O. Ampofo, Dr. J. Obeng, and Dr. M. Appiah, Tetteh Quarsh Memorial Hospital and Ministry of Health, Mampong-Akwapim, Ghana, West Africa.
Dr. T. Shehata, LPB, NIAMDD.

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Comparative Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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

Human red cell "sickling" and "desickling."

Sickle cell disease is a non-infectious hereditary anemia in which the disease process is well understood at the molecular level: the defective molecule has not only been identified as an abnormal hemoglobin (Hb S), but the abnormal interactions of this molecule can be explained. Observations reported from many laboratories are consistent with an aggregation mechanism due to hydrophobic interactions by deoxy Hb S, as postulated by Murayama. More recently, we have found in our laboratory that both aggregation and solubility of deoxy Hb S are inhibited by inorganic ions in the order of the classical Hofmeister series. We also found that red cells increase in volume upon "sickling." This change opposite in direction to published claims or predictions, is in the direction of the volume increase during sickling observed by Murayama in his high hydrostatic pressure experiment and with his thermodynamic prediction of the volume of activation. In collaboration with Dr. Oku Ampofo, Dr. J. Obeng, and Dr. M. Appiah in Ghana additional data are obtained supporting our previous finding of the effectiveness of oral carbamide in prevention of crisis and ameliorating the symptoms of sickle cell crises.

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 thermal aggregation of Hb S; and the volume of activation in the endothermic aggregation of Hb S.

3. To investigate further the effects of the constituents of the Hofmeister Series on the blockage and reversal of sickling using S hemolysate and S erythrocytes.

4. To study the effects of inhibitors of microtubule formation because:

   a. Hb S forms microtubule upon sickling.
   b. There is evidence for hydrophobic interactions being responsible for Hb S microtubule formation.
   c. Microtubules in general are formed by hydrophobic interactions.

5. To investigate a co-factor of S Hb aggregation.

6. Red cell volume increase on "sickling."

Methods Employed:

A pulse height analyzer was used to determine cell volume increase. Differential interference (Nomarski) optics microscopy using a chamber which is adequate for high resolution light microscopy for time lapse cinephotomicrography of sickling and unsickling process; in addition observations are recorded on magnetic video tape for future transcribing only the desired "strips" onto 16 mm film. Other methods are essentially the same as reported previously.

Major Findings:

Red cell volume increases from 10% to 30% upon "sickling." Sickle cell hemoglobin when deoxygenated has great propensity to aggregate; this aggregation can be inhibited by inorganic ions according to the classical Hofmeister series (Cl^- NO_3 Br^- I^- SCN^-). Essentially the same results were obtained by the thermodynamic solubility tests in a phosphate buffer. The solubility test was used to investigate the influence of monohydric and polyhydric alcohols on aggregation of sickle cell hemoglobin. It was found that the effectiveness of a series of polyhydric alcohols to promote aggregation increases as the number of hydroxymethyl groups about the asymmetric carbon atoms also influences the degree of aggregation.

Significance to NIAMDD Research:

Sickle cell anemia is a hereditary, hemolytic anemia -- an inborn error of metabolism, hence significant to the NIAMDD mission. My theory of hydrophobic interaction as the root cause of human red cell sickling has now been confirmed by nearly a dozen different laboratories. My theory has been tested at the bedside with success in Michigan as well as in Africa (oral urea therapy and oral urea prophylaxis), Sao Paulo, Brazil, and Havana, Cuba. When urea therapy has been administered with no intellectual understanding of the theory behind urea, as in the instance of NIH-NHLI cooperative study, this could lead to failure.

Proposed Course of Project:

Inorganic ions of Hofmeister series will be studied to find out whether or not any combination of two or more salts would yield an additive effect. If this would be true, then sodium iodide and sodium thiocyanate could be used with impunity (sodium thiocyanate would decrease the activity of the thyroid gland whereas sodium iodide will accerate same).

Publications:

Levine, A. S., Hasegawa, F. and Murayama, M.: Perturbants Affecting Gelation, Rates of Aggregation and Solubility of Sickle Cell Hemoglobin. In Hercules, J. I., Schechter, N., Eaton, W. A., and Jackson, R. E. (Eds.): Proceedings of the First National Symposium on Sickle Cell Disease. Wash., D.C., DHEW Pub. 75-723, 1974, pp. 147-149.

Levine, A. S. and Murayama, M.: Solubility of sickle cell hemoglobin: Inhibitors of the sickling process. J. Mol. Med. 1: 27-34, 1975.

REPORTED IN PRESS - 1975

Levine, A. S., Hasegawa, F. and Murayama, M.: The influence of solutes and solvent structure on gelation and aggregation of deoxy-sickle cell hemoglobin. J. Mol. Med. 1: 19-26, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-27008-17

PERIOD COVERED: July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies of metabolic activity in microorganisms

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

PI:	E. S. Kempner	Research Physicist	LPB NIAMDD
OTHER:	J. H. Miller	Chemist	LPB NIAMDD
	T. E. Shehata	Staff Fellow	LPB NIAMDD

COOPERATING UNITS (If any)

None

LAB/BRANCH: Laboratory of Physical Biology

SECTION: Comparative Physiology Section

INSTITUTE AND LOCATION: NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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

Studies of metabolic pathways in axenic cultures of microorganisms are continuing. The relationships between biochemically sequential reactions are being examined.

PHS-6040
(12-75)

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:

Previous studies of Euglena gracilis led to the conclusion that all macromolecules were bound in vivo to large organelles. The arrangement of enzymes is probably not a random attachment, but rather a spatial pattern related to the biochemical sequence of reactions that they catalyze. Upon disruption of cells, there is a release of those enzymes which were weakly bound, while others remain attached to mitochondria, lysosomes and other "particulates." The use of bifunctional reagents now offers the possibility of determining both the association of an entire pathway with a specific organelle and also the spatial arrangement of the individual enzymes of a biochemical sequence.

The synthesis of the amino acid tryptophan is accomplished via twelve enzymatic steps starting with erythrose-4-phosphate; the same pathway is found throughout the bacteria, algae and fungi. The first seven steps, up to chorismic acid, are common to other pathways which lead to phenylalanine, tyrosine, para-aminobenzoic acid and the isoprenoid quinones. In Euglena gracilis, this 12-step pathway is more highly organized than in any other species -- or at least can be extracted in more highly organized units. We have found that the first enzyme sediments with large cell particulates. Enzymes 2 through 6 are contained in a small complex of $1\text{-}2\times10^5$ MW. Enzyme 7 and 8 are extracted "unattached" (although reaction #8 is probably several biochemical steps and therefore may be a "complex" of enzymes). Enzymes 9 through 12 are joined in a single unit of 234,000 MW. These complexes are extractable and stable; it is possible that in vivo they may be even further co-joined. No other organism has been found to have such a high degree of organization in this pathway. It is even more remarkable that so many enzymatic steps should be contained within so small a set of molecular weights.

When cultures of Euglena gracilis in balanced growth are transferred from a minimal to a complex medium, our results show that only the mean cell volume changed, with little or no change in specific growth rate. Under our experimental conditions the mean cell volume of Euglena

gracilis is not uniquely determined by the specific growth rate. This is in contrast to the widely-accepted finding that in bacteria there is a simple relationship between cell size and the growth-supporting ability of the chemical environment. It seems possible that during cultivation in complex media, Euglena might have different regulatory mechanisms than those of the enteric bacteria. Environmentally induced variation in cell volume and composition are providing useful experimental approaches for studying growth and division of Euglena.

We previously reported that vitamin B_{12} starvation of Euglena was accompanied by cell enlargement and a gradual decrease in the specific growth rate until it finally stopped. Vitamin B_{12} deficiency appears to bring about the arrest of cell division of Euglena gracilis. Experimentally induced B_{12} starvation proved to be a useful technique to generate synchronous growth of Euglena. An important method of evaluating the synchronous growth of Euglena was provided by following the distribution of cell volume during the division cycle. We obtained a sequence of such distributions during synchronous growth of Euglena gracilis. During division, one peak is formed by newly-born cells, which translates and spreads as cells grow, while another peak is formed by cells which have not yet divided. The analysis of synchronous growth of Euglena gracilis is under study.

The histograms of DNA content of Euglena gracilis in steady state culture and vitamin B_{12} deficiency culture were measured by flow cytofluorometric technique and found to be consistent with chemical measurements. Synchronous culture permits more definitive studies of shifts in the distribution of DNA, in which the biochemical events required for cell division are presumably synchronized. With these techniques, we are studying the sequential changes during the division of Euglena.

Publications:

Kempner, E. S.: Properties of organized metabolic pathways. Subcellular Biochemistry. In press.

Miller, J. H. and Kempner, E. S.: The molecular biology of Euglena gracilis. X. Amino acid composition of protein. J. Protozoology. In press.

Shehata, T. E. and Marr, Allen G.: Effect of temperature on the size of Escherichia coli cells. J. Bacteriol. 124: 857-862, 1975.

ANNUAL REPORT OF THE
LABORATORY OF BIOPHYSICAL CHEMISTRY, NIAMDD

THE STRUCTURE AND ACTION OF PROTEINS

SICKLE-CELL HEMOGLOBIN: During the last year, the thermodynamic model for gelation of sickle-cell hemoglobin (HbS) has been extended and generalized. The new extensions facilitate both macroscopic and microscopic characterization of the gelling process using the results of readily performed measurements of sedimentation equilibrium and equilibrium oxygen binding. Analysis of data in the literature reveals, inter alia, that the tendency of HbS to gel is retained in large measure, even at fractional oxygen saturations exceeding 50%.

In collaboration with L. Fung and C. Ho of the University of Pittsburgh, the high-resolution proton NMR spectra of hemoglobin M Milwaukee has been measured and analyzed. The oxygen dependence of the ferric hyperfine shifted resonances, which monitors oxygen-linked structural charge in the vicinity of the ferric β-hemes, is inconsistent with a two-structure model of heme-heme interaction and supports the concept of direct ligand-linked interaction between subunits embodied in a sequential model. (Minton).

TRANSGLUTAMINASE AND TUMOR GROWTH: As we have reported previously, a correlation appears between the transglutaminase content of various organs and the extent of metastases into these organs in CAF_1 mice. We report now that intravenous injection of transglutaminase into mice greatly speeded up metastases of the Lewis lung tumor into the lungs. Also, immunization of the CDF_1 mice against guinea pig transglutaminase retarded the growth of the primary tumor. Retardation of the tumor and prolongation of the life of the mice was observed when mouse or rabbit antiserum (generated against guinea pig transglutaminase) was administered intraperitoneally to tumor-bearing CAF_1 or CDF_1 mice. These experiments suggest that there is a casual relationship between tumor proliferation and transglutaminase content. This is an analogous situation to the proliferation of granulation tissue (prompting our experimentation with tumor proliferation) which is impaired when plasma transglutaminase is lacking or speeded up by the administration of transglutaminase. (Laki, Csako, Yancey).

HOW PROLIFERATING TISSUES MAY AVOID IMMUNE SURVEILLANCE. THE ROLE OF TRANSGLUTAMINASE: According to current estimates, 1600 people in the United States are diagnosed every day to have cancer. This number would be much larger except for the immune surveillance systems which recognize malignant cells and mark them for destruction. It seems reasonable to consider also the converse of the surveillance. We propose that proliferating cells in order to survive must exercise "coverup" to escape surveillance.

It is generally accepted that not only malignant cells, but also all proliferating cells, have altered surface properties. In such a situation, it is imperative that the dividing cells not be marked as aliens. It is proposed that proliferating cells utilize transglutaminase in the "coverup" mechanism to attach proteins to their surface or connect surface proteins with isopeptide bonds. (Laki).

COMBINING FIBRINOGEN OR ACTIN TO THE SURFACE OF MALIGNANT PLASMA CELLS (MOUSE) BY MEANS OF TRANSGLUTAMINASE: In order to investigate what effect the addition of proteins to surface may have on the cell, labelled bovine fibrinogen and rabbit actin were prepared and attached to the surface of malignant plasma cells with the use of transglutaminase. To gain some idea of what proteins are involved in the binding of these proteins, the surface proteins were labelled with putrescine (C^{14}) with the use of transglutaminase. The labelling pattern was then compared to that one obtains when prior to putrescine labelling fibrinogen or actin was incorporated into the surface proteins. The experiments indicate that surface proteins of various molecular weight-ranges combined with fibrinogen. When the surface modified cells were implanted intraperitoneally into CAF_1 mice, these cells proved malignant. (Laki, Fesus, Yancey).

LAMPREY FIBRINOGEN: Detailed investigations were performed to characterize lamprey fibrinogen, the most primitive of the vertebrates, as to its kinetic-molecular properties and mode of action. Highly purified preparations via sedimentation-equilibrium gave a molecular weight of 365,000. The individual chains were also examined. The protein was also studied by differential scanning calorimetry. The results of clotting this protein by enzymes other than thrombin were examined by electron microscopy. (Gladner).

THE EFFECT OF FIBRINOPEPTIDE ON HEMOSTASIS: Canine fibrinopeptide A produced a dramatic hemostatic effect upon the bleeding time in the tail arterioles of the rat. Degradation of this peptide produced an active fragment of some eight amino acids from the N-terminal. The N-terminal threonine could also be removed without loss of activity.

A cooperative effect on hemostasis was evident when canine peptide A and ATP were combined. This enhancement of biological activity may be related to the participation of the adenyl cyclase system in the hemostatic process. During this hemostatic effect, the coagulation time remained unaffected in the presence of canine peptide A.

A decrease in the slope of the dose response curve of canine peptide A in the presence of propranolol, a receptor blocking agent, indicates that the involvement of the peptide in hemostasis may be at some step in the chain of events that links the receptor to the contractile mechanism.

An increase in the temperature of the system resulted in a marked decrease in the hemostatic effect which may be interpreted as an alteration in the active site of the receptors resulting in a decreased vasoconstriction at the site of the bleeding arterioles. (Osbahr).

THE COMBINATION OF ACTIN AND TROPOMYOSIN: A few years ago, it was reported from this laboratory that transglutaminase can use actin and tropomyosin as substrates and incorporate putrescine into these molecules. This time we can report that transglutaminase can connect these molecules into large polymers. Since our first report, it is now well established that actin and tropomyosin combine together. We now find that in this combination,

F-actin (in solution) acts as a template and determines the attachment of tropomyosin molecules; even when the two proteins are mixed in equal weights, actin prevents the formation of tropomyosin polymers. (Laki, Fesus).

Tropomyosin can be induced to bind to F-actin by myosin heads in the form of heavy meromyosin, myosin subfragment-1, and heavy meromyosin which has been modified by N-ethylmaleimide. Heavy meromyosin induces binding between tropomyosin and F-actin under conditions of low ionic strength (12mM KCl). This behavior is in contrast to that of troponin-I which induces binding between tropomyosin and F-actin only under conditions of higher ionic strength. (Kominz, Eaton).

HEMOGLOBIN AND COOPERATIVE EFFECTS: The oxidation of hemoglobin by hydroxylamine and oxygen (air) has been studied to explore the differences between the two reagents for the preparation of methemoglobin. (Simpson, Saroff).

Cooperative effects in hemoglobin are being evaluated from the standpoint of the quantitative contribution of the Bohr sites to the energy of interaction. (Saroff).

Aggregation effects and redistribution phenomena have been studied to examine their effects on cooperative interactions. (Saroff, Edelhoch, Ingham).

The slow degradation of egg albumin solutions and red cell hemolysates to generate free amino acids is being studied in an attempt to explain the phenomena. Evaluation of the distribution of amino acids, the absence of small peptides and inhibition by mercuric ions lead us to speculate that the degradation process may not be completely dependent upon contaminating enzymes. (Saroff).

FATTY ACIDS: Aggregation of the carboxylic acids has been studied by Raman spectroscopy indicating that solvent effects are critical in the interpretation of apparent aggregation. (Simpson).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 31000-16 LBC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Proteins, enzymes and peptides involved in blood coagulation

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

PI:	J. A. Gladner	Sr. Staff Investigator	LBC NIAMDD
OTHER:	P. A. Murtaugh	Res. Chemist	LBC NIAMDD

Other Cooperating Units at NIH:

S. I. Chung	Sr. Investigator	LB NIDR
M. S. Lewis	Investigator	LVR NEI

COOPERATING UNITS (if any)

J. E. Halver	Sr. Scientist	Coll. of Fisheries, Univ. of Wash., Seattle, Wash.
J. W. Donovan	Res. Chemist	West. Reg. Res. Labs., USDA, Berkeley, California

LAB/BRANCH

Laboratory of Biophysical Chemistry

SECTION

Physical Biochemistry

INSTITUTE AND LOCATION

NIAMDD-LBC, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	5	1

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

Detailed investigations were performed to characterize lamprey fibrinogen, the most primitive of the vertebrates, as to its kinetic-molecular properties and mode of action. Highly purified preparations via sedimentation-equilibrium gave a molecular weight of 365,000. The individual chains were also examined. The protein was also studied by differential scanning calorimetry. The results of clotting this protein by enzymes other than thrombin were examined by electron microscopy.

PHS-6040
(12-75)

Project Description:

Objectives: Major investigations center about the protein-protein interactions involved with and allied to blood coagulation. Particular orientation is directed to the biomolecular mechanisms of specific blood factors such as the thrombin action on fibrinogen and its other natural substrate, Factor XIII. These multiple reactions lead to fibrin formation, plus subsequent cross-linking (ligation) of fibrin monomers, to form the actual physiological material which constitutes a "blood clot". Understanding of these molecular biological mechanisms in human, as well as in lower vertebrates, may uncover valuable "clues" which can be utilized to practical advantages in the case of clotting disorders. Other investigations (preliminary) are directed to the enzymatic (natural) reversal of probably the key blood coagulation reaction, the cross-linking of fibrin monomers. The majority of all these reactions is enzymatically mediated.

Methods: Methodology stresses protein-enzyme fractionation, purification, and stringent characterization of all experimentally utilized materials. Enzyme mechanisms play a major role in these investigations; all aspects of such are employed. All methods of physical, analytical and enzyme biochemistry are utilized; for example, gel-electrophoresis, enzyme kinetics, radioactive incorporation assays, ultracentrifugation, automatic amino acid analysis, etc. Where needed, new and novel methods are devised.

Major Findings: Characterization and cross-linking of fibringens and fibrins of lower species: Past investigations have stressed elucidation of the cross-linking reactions of lamprey fibrinogen and particularly lamprey fibrin(s). However, due to the scarcity of these valuable materials, human and bovine thrombins and other factors were employed; these were actually hybrid experiments. In the past year, newer and more gentle methods were devised to isolate lamprey fibrinogen and thrombin in order to minimize degradation and obtain more stable molecules prior to definitive characterization. In addition, experiments were instituted to establish the mechanism of lamprey Factor XIII in fibrin cross-linking reactions. The following information has emerged:

(1) Since lamprey fibrinogen is the most primitive of the available vertebrate species, its importance in studies on the evolutionary aspects of proteins involved in coagulation is unique. Uncertainty of its molecular weight had made it difficult to reach meaningful conclusions regarding the structure of this molecule. This difficulty compounded elucidation of the mechanisms of its fibrin formation and polymerization. Subsequently, newer and more gentle fractionation methods resulted in the isolation of a more stable and amenable molecule. It can be stored at -80°C, thawed and utilized without degradation. The molecule can be lyophilized and redissolved in appropriate solvents, also without apparent degradation or denaturation. Prepared via these new methods, the molecule is of an exceedingly high order of purity. (Gladner and Chung).

(2) Sedimentation-equilibrium studies of the above material in various buffers and 6M guanidine utilizing data fitted via the MD-10 computer consistently yielded a molecular weight of 360,000. Within experimental limits, these results were identical with materials fractionated from different "batches" of lamprey plasmas collected over a period of 2-3 years and stored at -80°C. According to the literature, all fibrinogens are composed of 3 pairs of chains $(\alpha(A), \beta(B), \gamma)_2$. SDS-polyacrylamide gel electrophoreses of the reduced mixture yield molecular weights of 110,000, 78,000, and 50,000 for the $\alpha(A)$, $\beta(B)$, and γ respectively. These values, according to the above formulation, give a much higher molecular weight than is obtained via the much more critical and reliable sedimentation-equilibrium experiments. To overcome these discrepancies, the individual lamprey fibrinogen chains have been separated and purified. Molecular weight determinations via sedimentation-equilibrium methods are proceeding. Initial results indicate that the $\alpha(A)$ chain's molecular weight differs markedly from the value obtained by SDS-gel electrophoresis. These results and the prior utilization of partially degraded preparations may account for the many anomalies reported in the literature. (Lewis, Gladner, Chung).

(3) Past investigations have shown that only γ-chains were involved when lamprey fibrin was cross-linked by human thrombin activation of intrinsic Factor XIII in the presence of CA^{++} ions. Only the addition of human or bovine Factor XIII could demonstrate apparent cross-linking of the α-chains. Since these investigations demonstrate cross-linking sites in both the α- and γ-chains, experiments were performed to ascertain whether or not these

sites could be labelled. Although attempts were made to study the levels of lamprey Factor XIII in its plasma utilizing incorporation of radioactive putrescine into substrates, the results showed very low activity. This was in sharp contrast to the rapid dimerization of the γ-chains illustrated via SDS-gel electrophoresis. Dansyl cadaverine can inhibit cross-linking since, in the presence of Ca^{++}, Factor XIII will react it with the cross-linking sites; furthermore, the compound is fluorescent, thus labelling these sites. Cross-linking of the lamprey fibrin in the presence of dansyl cadaverine and Ca^{++} resulted in labelling of the γ-chain and its dimer. The α-chain appeared essentially unreactive in both polymer formation and dansyl cadaverine incorporation. These experiments suggest that only γ-cross-linking is essential to form a stable clot from lamprey fibrin. Detailed experiments to verify this are proceeding. (Chung, Gladner).

(4) Another study of the physical properties of our highly purified and lyophilized lamprey fibrinogen concerns parameters of its gross structure. The protein, as examined by differential scanning calorimetry, indicates 2 endotherms close to, but differing from, those reported for human fibrinogens. This may indicate important differences in the gross structure of this protein. (Donovan, Gladner).

(5) Investigations of the clotting of lamprey fibrinogen with enzymes other than thrombin, such as arvin, (a snake venom enzyme), and trypsin, have been carried out. In conjunction with the above, the resulting fibrins formed by either removal of the A or B peptide(s) have been studied by the electron microscope. In addition, certain lamprey fibrinogen preparations contain a clotting enzyme which does not appear to be thrombin on the basis of inhibitor studies. The electron microscope results have clearly shown a fiber of lamprey fibrin (with removal of either A or B peptide) which has a periodicity similar to human fibrin. This finding is of great interest considering that 400-500 million years separate the species. To date, it has not been possible to visualize lamprey fibrinogen with the electron microscope. (Murtaugh, Bladen, Gladner).

Significance to biomedical research and the program of the Institute: Detailed investigations of the characterization of blood coagulation systems of lower species may yield critical reactions and anomalies which may be masked in humans. Malfunctions or genetic throwbacks in human blood coagulation mechanisms may be discernible on the basis of these studies. Any information concerning clot formation is of vital importance since "heart attacks" and "strokes" are responsible for the majority of fatalities in the United States.

Proposed course of project: Emphasis will continue as has been proposed on the basis of this report. A major effort to expand these investigations employing all lamprey constituents (fibrinogen, thrombin, Factor XIII) will be stressed.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 31001-4 LBC

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mechanisms of Muscular and Cellular Contraction

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

PI:	David R. Kominz	Med. Director	LBC NIAMDD
OTHER:	Barbra Eaton	Staff Fellow	LBC NIAMDD
	Henry Wolff	Chemist	LBC NIAMDD

COOPERATING UNITS (if any)
Gerhard Steiger, UCLA Medical Center
Seth Goldstein, Mechanical Engineer, BEI, DRS, NIH

LAB/BRANCH
Laboratory of Biophysical Chemistry

SECTION
Bioenergetics

INSTITUTE AND LOCATION
NIAMDD-LBC, NIH

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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

Tropomyosin can be induced to bind to F-actin by myosin heads in the form of heavy meromyosin, myosin subfragment-1, and heavy meromyosin which has been modified by N-ethylmaleimide. Heavy meromyosin induces binding between tropomyosin and F-actin under conditions of low ionic strength (12mM KCl). This behavior is in contrast to that of troponin-I which induces binding between tropomyosin and F-actin only under conditions of higher ionic strength.

PHS-6040
(12-75)

Project Description:

Objectives:

To advance the understanding of contractility and its regulation.

Methods:

The unifying goal behind this multifaceted program of research has been to understand the energy-coupling mechanisms--in systems of purified contractile and regulatory proteins from skeletal muscle, in suspensions of myofibrils and various living or deal cells, and in the specialized geometrical array of glycerinated psoas muscle fibers. The effect of other muscle proteins on the binding between radioiodinated tropomyosin and F-actin has been determined in the preparative ultracentrifuge. Simultaneous measurement on myofibril suspensions of turbidity and ATPase activity have been carried out in the pH-stat as before. Major design changes have been incorporated in the system for fluorimetric ATPase monitoring of contracting glycerinated fibers, to avoid the necessity of a completely darkened room, and to allow moving the fiber from one solution to another.

Major Findings:

1. Tropomyosin could be induced to bind to F-actin by myosin heads in t the form of myosin subfragment-1 or of heavy meromyosin either before or after modification by N-ethylmaleimide. Heavy meromyosin induced binding between tropomyosin and F-actin under conditions of low ionic strength (12 mM KCl), whereas troponin-I required higher ionic strength to induce such binding.

2. With increasing ATP concentration, Triton X-100-treated myofibrils had their rigor bonds completely broken at a lower ATP concentration than that where the ATPase activity reached its maximum value.

3. When a fiber is severed and allowed to contract, its ATPase activity drops to about 75% of that at rest length.

Projects Proposed:

The principal investigator will continue these investigations in the Department of Physiology, University of Massachusetts School of Medicine, Worcester, Massachusetts.

Significance:

The binding studies suggest that the binding of myosin heads to actin causes a change in the conformation of the F-actin filament. This conclusion is inconsistent with the currently popular "steric blocking model" of

muscle regulation, and thus requires a reevaluation of earlier results from other laboratories as well as further experimental data.

Actin, found in the form of microfilaments in most cells, plays a poorly understood role in the mobility of cell membrane components and in the operation of the mitotic spindle and fission groove during cell division. There is an obvious need to understand its operation in normal and transformed cells. Recent work has suggested that unanchored myosin heads can induce contractility in structured actin--an attractive model for cellular function. The length-tension-ATPase monitoring system has been redesigned to allow careful study of such a system, which could be of broad value.

Publications:

1. Eaton, B. L., Kominz, D. R. and Eisenberg, E.: Correlation between the Acto-heavy Meromyosin ATPase and the Binding of Tropomyosin to F-actin: Effects of Mg^{++}, KCl, Troponin I, and Tropinin C. Biochemistry 14: 2718, 1975.

2. Eaton, B. L.: Tropomyosin Binding to F-actin Induced by Myosin Heads. Science, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 31002-5 LBC

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Intermolecular Interactions in Physical Biochemistry

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

PI: A. P. Minton Res. Chemist LBC NIAMDD

COOPERATING UNITS (if any)
Chien Ho, Prof., Dept. of Life Sciences, Univ. of Pittsburgh, Pittsburgh, Pa.
Leslie W.-M. Fung, Res. Assoc., Dept. of Life Sciences, Univ. of Pittsburgh, Pittsburgh, Pa.

LAB/BRANCH
Laboratory of Biophysical Chemistry

SECTION
Section on Macromolecules

INSTITUTE AND LOCATION
NIAMDD LBC NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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

During the last year, we have extended and generalized the thermodynamic model for gelation of sickle-cell hemoglobin (HbS). The new extensions facilitate both macroscopic and microscopic characterization of the gelling process, using the results of readily performed measurements of sedimentation equilibrium and equilibrium oxygen binding. Analysis of data in the literature reveals that the tendency of HbS to gel is retained in large measure even at fractional oxygen saturations exceeding 50%.

In collaboration with L. Fung and C. Ho of the University of Pittsburgh, we have measured and analyzed high-resolution proton NMR spectra of hemoglobin M Milwaukee. The oxygen dependence of the ferric hyperfine shifted resonances, which monitors oxygen-linked structural charge in the vicinity of the ferric β-hemes, is inconsistent with a two-structure model of heme-heme interaction and supports the concept of direct ligand-linked interaction between subunits embodied in a sequential model.

PHS-6040
(12-75)

Project Description:

Objectives:

1. To increase our knowledge about and understanding of the process by which sickle-cell hemoglobin aggregates; and to use this knowledge and understanding to develop chemical means for inhibiting the aggregation of sickle-cell hemoglobin *in vivo*, thereby alleviating symptoms of sickle-cell disease.

2. To ascertain the structural basis of cooperative ligand binding in hemoglobin.

3. To characterize the concentration-dependent properties of solutions of biological molecules in terms of specific (chemical) and non-specific (non-ideal) interactions between solute molecules.

Methods:

Theoretical studies employ methods of classical and statistical thermodynamics together with computer techniques for fitting theoretical model functions to experimental data. Experimental studies during the past year have utilized techniques of high resolution nuclear magnetic resonance spectroscopy. Planned studies will involve measurement of flotation equilibrium in the ultracentrifuge and the measurement of electric properties of solutions and cell suspensions.

Major Findings and Proposed Course of Studies:

The thermodynamic model for aggregation ("gelation") of sickle-cell hemoglobin which has been mentioned in the previous two annual reports of this project has been further extended and generalized:

a. New techniques have been developed for analyzing the concentration dependence of the oxygen-binding properties of HbS solutions and sickle RBC suspensions. Using these techniques, data in the literature have been interpreted in terms of the solubility of sickle-hemoglobin, its dependence upon the fractional oxygen saturation of the solution, and its dependence upon the ligand state of individual molecules. A major finding is that HbS in solutions approaching physiological concentration (>30 wt-%) appears to be substantally aggregated even at fractional oxygen saturations exceeding 50%. This suggests that a frequently proposed strategy for inhibiting sickling in sickle-cell patients by interfering with heme-heme interaction (and hence increasing oxygen affinity) is not likely to be successful. A second finding of some importance is that singly oxygenated HbS appears to have significantly less tendency to aggregate than does completely deoxygenated HbS, indicating that a substantial structural change is associated with the binding of a single molecule of oxygen to Hb.

b. A method has been developed for taking into account the pronounced non-ideality of concentrated hemoglobin solutions when analysing the thermodynamics of sickle-cell hemoglobin aggregation. This allows a more quantitative approach than has heretofore possible and, with the use of non-ideality data from non-aggregating hemoglobin solutions, permits a simple yet fairly accurate description of the equilibria between states of aggregation in a gelling HbS solution.

In collaboration with C. Ho and L. Fung of the University of Pittsburgh, the proton NMR spectra of hemoglobin M Milwaukee have been measured and analysed. The β-chains of Hb M Milwaukee contain permanently oxidized heme groups due to the presence of a glutamate residue in place of valine at the βE11 position in this mutant. Various features of the spectra indicate that the mechanism of heme-heme interaction in Hb M Milwaukee is qualitatively similar to that in normal hemoglobin A, even though only the ferrous α chain can bind oxygen. Of particular interest is the dependence of the paramagnetically shifted proton resonances upon the degree of saturation. These resonances monitor structural changes in the vicinity of the ferric β-hemes which are associated with the oxygenation of the α-hemes. The results are clearly inconsistent with a two structure model for hemoglobin and support the notion of direct ligand-linked interactions between subunits which is embodied in a sequential model for heme-heme interaction.

During the coming year we propose the following studies:

1. Continuation of the thermodynamic and structural characterization of ligand binding to the valence hybrid hemoglobins as a probe of the mechanism of heme-heme interaction in hemoglobin.

2. Exploration of the electrical properties of concentrated HbS solutions and cell suspensions; investigation the utility of measurements of permittivity and conductivity as monitors of HbS aggregation and/or cell sickling (in collaboration with J. Pumphrey).

3. Analysis of the dependence of the heat of binding of 2,3-diphosphoglycerate to hemoglobin upon the fractional oxygen saturation of hemoglobin (in collaboration with D. Nelson).

Significance to Biomedical Research and the Programs of the Institute:

The extensions of the thermodynamic model for HbS gelation reported here constitute a substantial step forward in our understanding of HbS aggregation under physiological conditions. In addition, they have provided simple techniques for the characterization of gelling properties which may be readily understood and utilized by the experimentalist or clinician without reference to thermodynamics or recourse to complex mathemetical calculations.

The NMR study of heme-heme interaction in hemoglobin M Milwaukee has yielded what my collaborators and I feel to be the most direct and least equivocal information on the role of protein structure in heme-heme interaction which has so far been reported. The experimental and analytical methods may be applied to other proteins, including enzymes, which bind ligand (reactant) cooperatively.

Publications:

1. Minton, Allen P.: Chemical inhibition of sickle-cell hemoglobin gelation: A thermodynamic analysis. J. Mol. Biol. 95: 289-307, 1975.

2. Minton, Allen P.: Relations between oxygen saturation and aggregation of sickle-cell hemoglobin. J. Mol. Biol. 100: 519-542, 1976.

3. Fung, Leslie W.-M., Minton, Allen P. and Ho, Chien: Nuclear magnetic resonance study of heme-heme interaction in hemoglobin M Milwaukee: Implications concerning the mechanism of cooperative ligand binding in normal hemoglobin. Proc. Nat. Acad. Sci. U.S.A. 73: 000, 1976.

4. Minton, Allen P.: Quantitative relations between oxygen saturation and aggregation of sickle-cell hemoglobin: Analysis of oxygen binding data. In Hercules, J., Schechter, A., Cottam, G. L. and Waterman, M. (Eds.): Molecular and Cellular Aspects of Sickle-Cell Disease. Dept. of H.E.W., 1976, in press.

5. Minton, Allen P.: Non-ideality and the thermodynamics of sickle-cell hemoglobin gelation. J. Mol Biol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 31003-02 LBC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The action of thrombin on fibrinogen and related blood clotting phenomenon and mechanisms.

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

PI: A. J. Osbahr Res. Chemist LBC NIAMDD

COOPERATING UNITS (If any)

None

LAB/BRANCH

Laboratory of Biophysical Chemistry

SECTION

Bioenergetics

INSTITUTE AND LOCATION

NIAMDD NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	none

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

The important physiological role of the peptides released from fibrinogen is being investigated. These peptides produce strong hemostasis in rat tail arterioles and induce hypertension after intravenous injection into the rat. The fibrinopeptides also act on the contractile proteins by increasing both the rate of superprecipitation and the hydrolysis of ATP.

The effect of structural modification upon the thrombin-induced conversion of fibrinogen to fibrin is being studied as a model for better understanding of the molecular aspects of the action of thrombin on fibrinogen.

PHS-6040
(12-75)

Project Description:

Objectives:

An improved understanding of the structural basis for the enzymatic action of thrombin on fibrinogen, and for the physiological action of the peptides released from fibrinogen during blood clotting, in order to gain insights into the possible role of these peptides in hemostasis, vasoconstriction and pulmonary hypertension.

Methods:

Homogeneity and purity of the protein and peptide systems were evaluated with ultracentrifugation and regular and SDS gel electrophoresis. The peptide structure and composition were analyzed by high voltage electrophoresis, liquid column chromatography and spectrophotometric analysis.

Proteolytic enzymes were employed for "active site" determinations. Physiological monitoring was performed on rats by means of intracarotid cannulation. The hemostatic effect was followed by microscopic visualization of bleeding arterioles of scarified rat tail.

Major Findings and Proposed Course of Study:

Peptide A from canine fibrinogen produced a marked hemostatic effect upon the bleeding arterioles of scarified rat tail. Bleeding time was decreased from 110 sec. to 10 sec. at tenth milli molar concentration of canine A peptide. The addition of ATP to the incubation mixture of canine peptide A produced a further decrease in the bleeding time and therefore

enhanced the hemostatic effect produced by the peptide. It is possible that the adenyl cyclase system participates in this hemostatic effect.

Degradation of the canine A peptide was performed and the peptide reduced to half its size via chymotryptic action. An eight amino acid fragment from the N-terminal retained all of the physiological activity. The N-terminal threonine residue of this active fragment could also be removed without resulting loss of activity.

During this hemostatic effect in the presence of canine peptide A, the coagulation time is more prolonged than the bleeding time and was unaffected by the presence of the varying concentrations of the peptide. This suggests that the coagulation process could not be primarily responsible for the observed hemostatic process.

A decrease in the slope of the dose response curve of canine peptide A in the presence of propranolol, a receptor blocking agent, indicates that the receptor population is partially inactivated. This suggests an alteration of one of the steps in the chain of events that link the receptor to the contractile mechanism.

It is possible that the peptide acts in this hemostatic effect by altering the muscle receptor site with a resultant gain in biologic activity and terminating in increased vasoconstriction at the site of the bleeding arterioles.

In an attempt to characterize the molecular nature of the peptide on the receptors the physical method of temperature was used. An increase of ten degrees in temperature inhibited the hemostatic effect and a marked decrease in the slope of the peptide dose response curve resulted.

An interpretation of the heat response is that heat may have caused an alteration in the structure of the active site of the receptors. These mild conditions suggest that the heat labile receptors may be composed entirely or in part of protein molecules which undergo irreversible heat denaturation.

Significance of Biomedical Research and the Program of the Institute:

Our studies indicate a relationship between the peptides from the blood clotting protein fibrinogen and the physiological processes in blood circulation. It is hoped that investigation of these physiologically active peptides may result in a deeper understanding of the mechanisms associated with hemostasis, vasoconstriction and blood coagulation; and could provide clues to the etiology of certain diseases associated with high blood pressure such as respiratory distress syndrome. The presence of fibrinopeptide may represent a possible agent for such vasoconstriction. The more we know about the physiological activity of these peptides, the easier could be the task of sorting out some lesser known mechanisms

involved in the related diseases.

The nonenzymatic polymerization of fibrinogen may be used as a model to investigate the thrombin induced polymerization of fibrinogen. Methylation induced polymerization of fibrinogen suggests that the first step in the mechanism of clot formation is the removal of the negative replusion at the ends of two of the fibrinogen chains without peptide material being necessarily simultaneously released.

Proposed Course of Project:

Further investigation of the modified fibrinogens with proteolytic enzymes to establish the mechanism of release of fibrinopeptides.

The action of thrombin on the modified fibrinogens will be investigated in an attempt to release modified fibrinopeptides.

Further chemical modification of specific functional groups in fibrinogen and the fibrinopeptides will be explored to gain greater insight into the relationship between structure, biological activity and enzymatic specificity.

The physiological role of the native and modified peptides from fibrinogen will be further investigated to establish the role of receptor sights in the mediation of the fibrinopeptide interaction with the contractile mechanism enabling vasoconstriction to occur.

Publication:

1. Osbahr, A. J.: Structure-Activity Relationships of the Vasopressor Activity of Canine Peptide-A from Fibrinogen. Biochimica et Biophys. Acta 386: 373-382, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 31004 05 LBC

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Elucidation of the Structure and Interactions of Biologically Important Macromolecules

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

PI: H. A. Saroff	Sci. Dir.	LBC NIAMDD
OTHER: R. B. Simpson	Res. Chemist	LBC NIAMDD

COOPERATING UNITS (if any)

Clinical Endocrinology Branch, NIAMDD

LAB/BRANCH
Laboratory of Biophysical Chemistry

SECTION
Macromolecules

INSTITUTE AND LOCATION
NIAMDD-LBC, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

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

The quantitative relationship between the Bohr sites and the energy of interaction of hemoglobin is being studied. With these data it is possible to refine our model for the action of hemoglobin by assigning proper weights to the contribution of the Bohr effect in generating the cooperativity of hemoglobins.

Oxidation of hemoglobin to methemoglobin is being studied under a variety of conditions with different reagents.

Cooperative binding systems derived from aggregation and redistribution are under study.

The slow generation of amino acids and possibly peptides by solutions of egg albumin and hemoglobin is being studied.

PHS-6040
(12-75)

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 accummulation of data on the state of the molecule with respect to its environment. Gross features of structures as well as fine detail may be observed by studies.

Methods:

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 molecules, 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:

A. Aggregation of carboxylic acids: The binding of fatty acids to albumin is of interest in the transport of these substances in vivo.

Interpretation of data on binding requires knowledge of the state of aggregation of these carboxylic acids in solution. Studies of the Roman spectra of these substances in water has revealed that solvent (water) effects are important in interpreting conductivity data employed to evaluate aggregation constants. (Simpson)

B. Oxidation of hemoglobin: The kinetics of the oxidation of hemoglobin by air (oxygen) and by hydroxylamine are under continued study. In the study of the reaction of hydroxylamine with hemoglobin, the product of this reaction showed an electron paramagnetic resonance spectrum very similar to that of denatured hemoglobin complexed with nitric oxide. A plausible mechanism for the production of nitric oxide is $H_2NOH + 3Fe^{+3} \rightarrow NO + 3Fe^{+2} + 3H^{+}$. (Simpson, Saroff)

C. Action of hemoglobin: Studies are being continued to evaluate the quantitative contribution of the pH depended (Bohr) sites to the interaction energy responsible for the cooperative effect in hemoglobin. Our predictions on the qualitative relationship between the cooperative effect and the Bohr sites have been confirmed by recent publications on hemoglobin. (Saroff)

D. Slow generation of free amino acids from hemoglobin and egg albumin: Sterile solutions of crude and crystalline preparations of egg albumin as well as sterile solutions of red cell hemolysates generate significant amounts of free amino acids on standing for ten to thirty days. Purified hemoglobin A, the crystalline mercury dimer of bovine serum albumin, and purified thyroglobin generate no amino acids on standing (less than 2×10^{-9} mole of amino acid per ml of 1 to 5% protein solution). Quantitative evaluation of the distribution of amino acids, the absence of small peptides, and inhibition by mercuric ions lead us to speculate that the slow degradation process may not be completely dependent upon contaminating enzymes. Proteolytic enzymes reported in red cell hemolysates include the ubiquitous cathepsins (associated with the stroma) and a number of peptidases. We have found no references to proteolytic enzymes in egg white. (Saroff)

E. Cooperative effects: We are analyzing aggregation and redistribution phenomena to examine their effects on cooperative interactions. In conjunction with this effort we have proposed an association reaction to explain the binding of ANS (8-anilino-1-naphithalensidfonic acid) to hCG and hLH. (Saroff, Edelhoch and Ingham).

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 extrapolation to the biological system. Studies on the binding properties of protein systems related to certain hormones provide insight to possible modes of cooperative effects in hormone action.

Publications:

1. Simpson, R. B.: Conductivity Anomalies of Aqueous Carboxylic Acids Solutions. Dimerization or Effect of Solvent Medium? J. Phys. Chem. 79: 1450-1455, 1975.

2. Ingham, K. C., Saroff, H. A. and Edelhoch, H.: Ligand-Induced Self-Association of Human Luteinizing Hormone. Negative Cooperativity in the Binding of 8-Anilino-1-naphthalenesulfonate. Biochemistry 14: 4745-4750, 1975.

3. Ingham, K. C., Saroff, H. A. and Edelhoch, H.: Ligand-Induced Self-Association of Human Chorionic Gonadotropin. Positive Cooperativity in the Binding of 8-Anilino-1-naphthalenesulfonate. Biochemistry 14: 4751-4758, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 31006-07 LBC

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Transglutaminase and tumor growth

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

PI:	K. Laki	Chief, Lab. Biophys. Chem.	LBC-NIAMDD
OTHER:	E. F. Wilson	Chemist	LBC-NIAMDD

COOPERATING UNITS (if any)

S. I. Chung	Sr. Investigator	LB NIDR
S. T. Yancey	Biol. Lab. Tech.	LCP NCI

LAB/BRANCH
Laboratory of Biophysical Chemistry

SECTION
Physical Biochemistry

INSTITUTE AND LOCATION
NIAMDD LBC NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/2	1.0; 1/4	1/4

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

The experiments reported show that intravenous administration of transglutaminase (guinea pig liver) promoted tumor metastases in mice and that antibody against transglutaminase, when administered to tumor-bearing mice, retarded tumor growth and prolonged life of the animals.

PHS-6040
(12-75)

Project Description:

Objectives: Earlier, we found a correlation between transglutaminase content of tissues and the growth of tumor in those tissues. This time we inquired if this correlation also means a cause and effect relationship.

Major Findings: We report now that intravenous injection of transglutaminase into mice greatly speeded up metastases of the Lewis lung tumor into the lungs. Also, immunization of the CDF_1 mice against guinea pig transglutaminase retarded the growth of the primary tumor. Retardation of the tumor and prolongation of the life of the mice were observed when mouse or rabbit antiserum (generated against guinea pig transglutaminase) was administered intraperitoneally to tumor-bearing CAF_1 or CDF_1 mice. These experiments suggest that there is a casual relationship between tumor proliferation and transglutaminase content. This is an analogous situation to the proliferation of granulation tissue (prompting our experimentation with tumor proliferation) which is impaired when plasma transglutaminase is lacking or speeded up by the administration of transglutaminase.

Test tube experiments show that the antiserum produced in the mice against transglutaminase is a good inhibitor, not only by the guinea pig liver enzyme, but also the enzyme in mouse liver homogenate. It seems reasonable to assume that the immune serum (but not normal serum) inhibits tumor proliferation because of the presence of antibody against transglutaminase.

Significance to biomedical research and the program of the Institute: We hope that these studies will shed light, not only on certain aspects of tumor growth, but also on tissue proliferation in general.

Proposed course of project: We intend to carry out similar studies with purified antibody preparations to ascertain that the effect is definitely due to the antibody against transglutaminase.

Publications:

1. Laki, K., Csako, G., Yancey, S. T. and Wilson, E. F.: A possible role of transglutaminase in tumor growth and metastases. Proc. Symp. honoring Prof. Albert Szent-Györgyi, Boston, Oct. 16-17, 1975. In press.

2. Laki, K., Csako, G., Yancey, S. T. and Suba, E.: Proc. 24th Annual Wayne State Univ. Symp. on Blood, Jan, 22-23, 1976, Detroit, Michigan. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 31008-01 LBC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

How proliferating tissues may avoid immune surveillance. The role of transglutaminase.

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

PI: K. Laki Chief, Lab. Biophys. Chem. LBC NIAMDD

COOPERATING UNITS (If any)

S. Yancey Biol. Lab. Technician LCP NCI

LAB/BRANCH

Lab. of Biophysical Chemistry

SECTION

Physical Biochemistry

INSTITUTE AND LOCATION

NIAMDD-LBC NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/2	

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

It is proposed that because of a coverup mechanism involving transglutaminase, proliferating cells can avoid immune surveillance.

PHS-6040

Project Description:

Objectives: According to current estimates, 1600 people in the United States are diagnosed every day to have cancer. This number would be much larger except for the immune surveillance systems which recognize malignant cells and mark them for destruction. It seems reasonable to consider also the converse of the surveillance. We propose that proliferating cells in order to survive must exercise "coverup" to escape surveillance.

It is generally accepted that not only malignant cells, but also all proliferating cells, have altered surface properties. In such a situation, it is imperative that the dividing cells not be marked as aliens. It is proposed that proliferating cells utilize transglutaminase in the "coverup" mechanism to attach proteins to their surface or connect surface proteins with isopeptide bonds.

Major Findings: We have been trying various inhibitors of transglutaminase in order to see if during retarded tumor growth, a defense mechanism develops against the tumor.

In our earlier experiments, we have implanted polylysine, an inhibitor of transglutaminase together with the YPC-1 cells, hoping that the prolonged presence of the malignant cells may activate a defense mechanism. This we could not notice. Antibody against transglutaminase appears more promising in this regard. Active immunization caused a retardation of the growth of the subcutaneous Lewis lung tumor and all the immunized mice exhibited tumor necrosis. When serum of the immunized mice was mixed with the implanted Lewis lung tumor cells and subsequently injected peritoneally, a diminished incidence of tumor was observed in the antibody-treated animals. Current

experiments indicate that antibody generated in rabbits (against guinea pig transglutaminase) applied similarly with YPC-1 tumor to CAF_1 mice has a similar effect.

These experiments suggest that the outcome of tumor proliferation may depend on the interplay of immune surveillance and the coverup mechanism, and that the "roundabout" immunization with antibody against transglutaminase may be beneficial.

Significance to biomedical research and the program of the Institute: The investigations may lead to a better understanding of cell proliferation.

Proposed course of project: We intend to use purified antibodies against transglutaminase to see if the above propositions can be further substantiated.

Publications:

1. Laki, K. and Ladik, J.: Protein Energy Converters. In Andre, J., Ladik, J. and Delhalle, J. (Eds.): Electronic Structure of Polymers and Molecular Crystals. New York, Plenum Press, 1975, pp. 681-698.

2. Laki, K.: Actin as an ancient nucleotide-binding protein. Int. J. Quantum Chem., Quantum Biol. Symp. No. 2, 297-305, 1975.

3. Laki, K.: Faktor XIII und Metastasis. Thromb. Diath. Haemorrh. In press.

4. Laki, K. and Ladik, J.: A Note on the "Electronic Theory" of Cancer. Int. J. Quantum Chem. In press.

5. Laki, K.: Twenty Years A-Growing with Albert Szent-Györgyi. Int. J. Quantum Chem. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 31009-01 LBC

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Combining fibrinogen or actin to the surface of malignant plasma cells (mouse) by means of transglutaminase

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

PI:	K. Laki	Chief, Lab. Biophys. Chem.	LBC-NIAMDD
	L. Fesus	Visiting Fellow	LBC-NIAMDD

COOPERATING UNITS (if any)

S. T. Yancey	Biol. Lab. Tech.	LCP NCI
S. I. Chung	Sr. Investigator	LB NIDR

LAB/BRANCH

Laboratory of Biophysical Chemistry

SECTION

Physical Biochemistry

INSTITUTE AND LOCATION

NIAMDD LBC NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1/4; 1/4	1/2

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

It was found that tissue transglutaminase modifies surface of proliferating cells by attaching fibrinogen (or actin) to surface proteins with isopeptide bonds.

PHS-6040
(12-75)

Project Description:

Objectives:

The experiments are aimed to investigate what effect the insertion of proteins into the membrane of cells has on the properties of cells. For this purpose, we chose malignant plasma cells of the mice (CAF_1) and used transglutaminase to attach bovine fibrinogen and rabbit actin to membrane proteins.

Major Findings:

In one set of experiments, the two proteins were labelled by introducing labelled iodine into tyrosine residues (lactoperoxidase technique) or onto the lysine residues (Bolton-Hunter reagent). The experiments show that these two proteins attach to the membrane of the plasma cells. When the cells are broken up and the membrane debris studied, we find that about 10^6 fibrinogen molecules bind to a plasma cell. From the experiments, we can surmise that in case of lysine labelled fibrinogen binding to membrane proteins occurs through the cross-bonding sites of the γ-chain of fibrinogen.

In order to gain some idea what surface proteins are involved when fibrinogen binds to the surface, we first labelled surface with putrescine (C^{14}), again using transglutaminase. The membrane debris after denaturation was examined on SDS gels and the radioactive peaks ascertained. These were then compared to those obtained in similar experiments in which, prior to putrescine incorporation, actin or fibrinogen (not labelled) were incorporated into the membrane proteins. The comparison revealed that fibrinogen binds to many surface proteins belonging to various weight classes. The plasma cells from these experiments were obtained from ascites fluid freed from red cell admixture and then washed with saline. In spite of the foreign proteins incorporated into the surface of these plasma cells, these remained viable and killed the animals in the expected time (2 weeks) when implanted intraperitoneally.

Significance to biomedical research and the program of the Institute:

This may be a possible new approach to the study of surface proteins of cells.

Proposed course of project: We are going to extend the procedure to platelets.

Publications: None.

ANNUAL REPORT OF THE LABORATORY OF MOLECULAR BIOLOGY

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

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, 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, characterization of protein-DNA interaction in chromatin, investigation of protein structure by X-ray diffraction, and theoretical studies of muscle contraction, free energy transduction, and of nerve membrane characteristics. Major advances in several of these areas have been made by members of the laboratory during the past year.

Chromatin Structure and Function

The broad purpose of this project is to understand the way in which chromatin, the DNA-protein complex in the nucleus of higher organisms, operates to establish and maintain regulated cell function. Investigations are in progress to understand chromatin structure, i.e. the specific organization of proteins and DNA within the complex, and the relationship of this structure to biological functions. The biological function of present interest is control of gene expression: the proteins of chromatin serve to block certain gene sequences from being transcribed into messenger RNA. This is believed to form the chemical basis of cell differentiation. One test system being investigated is globin messenger RNA synthesis in chromatin from avian reticulocytes. The goal is to isolate the biochemical factors in this chromatin responsible for maintaining globin message synthesis, and to understand how they affect chromatin structure to carry out their function. Other related studies are concerned with the folding of DNA by chromatin proteins, the physical arrangement of proteins on the globin gene, and the effects of these proteins on the transcription process. (Felsenfeld, Camerini-Otero, Melchior, McGhee, Sandeen, Simon, Sollner-Webb, Williamson and Zasloff).

Replication of Colicin E1 Plasmid DNA

Replication of closed-circular colicin E1 plasmid (Col E1) DNA can be initiated and completed in extracts of *Escherichia coli*. The major products of *in vitro* replication are completely replicated molecules and a unique type of early replicative intermediate containing a newly synthesized DNA fragment(s) in a small replication loop. The fragment has an average length of approximately one fifteenth of the unit length of the plasmid molecule and has a sedimentation constant of approximately 6S. The replicated region of the intermediate consists of either one double-stranded branch and one single-stranded branch or two double-stranded branches. These intermediates

accumulate in a reaction mixture containing 10% glycerol. Synthesis of the intermediates is inhibited by rifampicin but most of the intermediates can complete replication in the presence of rifampicin. We have studied the synthesis and fate of 6S DNA fragments formed on the parental heavy (H) strands and those formed on the parental light (L) strands of early replicative intermediates. The results show that the first synthesis of a DNA fragment is initiated at a specific site on the H strand and depends on the function of E. coli DNA-dependent RNA polymerase. Subsequent synthesis of the DNA fragment on the L strand does not involve the RNA polymerase.

A direct proof of the attachment of RNA to 6S L-DNA was shown by the transfer of ^{32}P-label in the DNA derived from [α-^{32}P]dNTPs to one of 2'(3') NMP after alkaline hydrolysis of 6S L-DNA. The base sequence at the RNA-DNA junction is specific but the specificity is not absolute. More than one third of the transfers are dT to rG and no significant transfer to rA was observed. The results indicate the presence of a preferred transition point as well as ambiguity at a single transition point. Preincubation of Col El DNA with purified bacterial RNA polymerase allowed some synthesis of Col El DNA in the presence of rifampicin. Some RNA synthesized by RNA polymerase was found to be attached to 6S L-DNA, indicating that RNA synthesized by bacterial RNA polymerase contains the primer for synthesis of the DNA.

In vitro Col El DNA replication can be carried out by bacterial functions without participation of any plasmid functions. Studies on necessary bacterial functions have been continued. DnaC(D) gene function was shown to be necessary for synthesis of 6S H-DNA but not of 6S L-DNA.

A cleavage map of Col El DNA by restriction enzymes was constructed. The region involving the origin of replication was finely mapped and the fragments which include the origin of replication were isolated for analysis of the base sequence.

With the in vitro system, the mechanisms of segregation of daughter molecules upon completion of replication of circular molecules can be studied. In collaboration with Y. Sakakibara (NIH, Japan) we studied mechanism of formation of cantenanes which consist of two interlocked circular molecules. It is concluded that two daughter molecules or a cantenane are formed as the alternative products upon completion of replication of a circular molecule. (Tomizawa, Bird, Itoh, Ohmori).

Studies on Integrative recombination of phage λ in a cell-free system

A novel assay has been developed for studying this site-specific recombination in vitro. The substrate (developed by H. Nash) is a λ DNA containing two sets of attachment sites; recombination excises the fragment between the sites. The assay looks at the sizes of restriction fragments produced by the Eco R1 enzyme. The recombined products have different fragment sizes, as measured by electrophoresis in agarose gels, from the starting DNA.

Using this assay, the following results have been obtained:

1) Covalently circular DNA is the only effective substrate. Linear DNA is inactive; nicked circular DNA works only if it can be sealed by DNA ligase in the reaction mixture.

2) Both recombination products (the excised fragment and the remaining λ DNA) are recovered in equal yield.

3) Both recombination products are recovered largely in the form of covalent circles, even when DNA ligase in the cell extracts is inhibited by NMN. This raises the possibility of a ligase-independent sealing step as a coupled part of the recombination process.

4) If the products are not treated with restriction enzyme, most of the excised fragment is recovered as circles interlocked (catenated) with the circular λ DNA product. This result implies that recombination occurs while the substrate DNA is supercoiled. (Mizuuchi, Gellert, Nash, O'Dea).

In vitro DNA Replication Catalyzed by purified E. coli proteins

Genetic studies by others of E. coli mutants temperature sensitive for DNA synthesis have resulted in the identification of 11 genes whose functions are required for chromosome replication. Some of these proteins are also required for the conversion of single-stranded circular phage DNAs to duplex DNA. The proteins catalyzing these reactions using fd, ST-1 or ØX174 phage DNA templates have been purified from uninfected E. coli.

The dnaZ gene product is required in all three of these DNA synthesizing systems. Using this requirement it has been purified about 5000-fold. It functions in the elongation of RNA or DNA-primed single-stranded DNA catalyzed by DNA polymerase III in conjunction with two other E. coli protins referred to as DNA elongation factors I and III in a reaction requiring ATP or dATP. It also functions in similar reactions catalyzed by DNA polymerase II in combination with E. coli DNA binding protein and DNA elongation factors I and III. Elongation factors I and III have been purified by their requirement in this reaction but have not been identified genetically yet. (Wickner).

Novel Recombination Systems

A gene for chloramphenicol-resistance (cam), derived from an R-factor, is present on a sequence of DNA which is translocatable to diverse replicons (e.g. coliphages P1 and λ and Escherichia coli chromosome itself) in the absence of known recombinational functions or sequence homology. The basis of this extraordinary recombinational activity is being studied by genetic analysis and electron microscopy of heteroduplex DNA.

A second feature of this "translocon" is that it gives rise to deletions at high frequence resulting in the loss of the cam gene from the sequence. Some of the deletions are wholly within the translocon while others extend into the phage DNA. Particular attention is being given to a phage which

does not lose the cam gene at high frequency. This phage is deleted for a portion of the phage chromosome adjacent to the translocon. The extent to which the deletion extends into the translocon is being studied. (Rosner).

Genetics and structure of the oncogenic virus, SV40

It is the long range goal of this project to discover how SV40 causes transformation. SV40 is a small DNA virus of the papova virus group. Papoviruses have been implicated in cancer and progressive multifocal leukoencephalopathy. Current topics of interest are (1) the cell cycle in normal and SV40 transformed cells, (2) the origin and structure of the tumor specific transplantation antigen, (3) the structure of SV40 chromatin, (4) regulation of the synthesis of SV40 tumor antigen, (5) the structure and function of the tumor antigen, and (6) the mode of DNA replication in normal and transformed cells. (Martin, Anderson, DiLauro, Brockman, Stein, Edwards).

Mammalian Enzymes Involved in Repair or Replication

The aim of this project is to identify and characterize enzymes involved in the repair and replication of DNA in higher organisms. Currently we are: 1) Examining the number and characteristics of DNA ligases in rat liver, 2) Characterizing a DNA kinase of unusual specificity also from that source. (Zimmerman and Levin).

Structural Studies on Polynucleotides

The aim of this project is to obtain structural information on polynucleotides and to provide insights into the factors which control structure, principally through X-ray fiber diffraction and model building studies. Current areas include 1) the structure of the polyU-Spermine complex, 2) prediction of structural parameters from observed helical parameters, 3) the structure of a polynucleotide-like helical complex adopted by a monomer (G-5'-P). (Zimmerman).

Biologically active Messenger RNA for Human Placental Polypeptide Hormones

The long range purpose of this project is to understand regulation of human placental polypeptide hormone synthesis at the levels at transcription and translation, and to examine the mechanism by which one of these proteins (the α subunit of human chorionic gonadatropin) is produced ectopically by several cell lines derived from human cancers.

Our current approach to examine the biological activity of placental mRNA in cell-free systems for protein synthesis. The poly(A) containing fraction of total placental RNA directs the synthesis of two polypeptides precipitable by antiserum directed against human placental lactogen (hPL). Electrophoresis on SDS polyacrylamide gels reveals that one product comigrates with authentic hPL, while the other migrates slightly slower and has an apparent molecular weight about 3000 units larger. We are presently investigating the possible precursor-product relationship of the two polypeptides.

Protein Chemistry of Polypeptide Chain Elongation Factors

We have compared the tryptic peptide maps of eukaryotic elongation factor 2 (EF-2) from rat liver and from rabbit reticulocytes with that of the analogous prokaryotic factor (EF-G) from E. coli. The differences between the maps of either EF-2 and that of EF-G are extensive. All three factors require at least one free sulfydryl group for activity. We have labeled the cysteines of EF-G and of rat liver EF-2 with ^{14}C ethyleneimine in order to identify a potentially conserved active site. The labeled peptides are now being purified prior to determination of their amino acid sequence.

All preparations of EF-2 tested to date are ADP-ribosylated in the presence of diphtheria toxin and NAD^+ and are thereby inactivated. EF-G is neither inactivated nor ADP-ribosylated by toxin and NAD^+. We previously sequenced the site of potential regulation around the covalently attached ADP-ribose on EF-2. The ADP-ribose is linked to an unusual amino acid (X) which has not been found previously in proteins. Free X was isolated following acid hydrolysis of a tryptic peptide and X-ribose was isolated following enzymatic degradation, but we have not yet identified the unknown residue. This year we have purified a tryptic peptide containing X from native EF-2 which has not been ADP-ribosylated. This peptide has the same amino acid composition as that containing X-ribose. It should now be possible to isolate sufficient free X by mild or enzymatic degradation to study the compound by gas chromatography-mass spectrometry and perhaps by NMR. (Maxwell, Tsai and Robinson).

Studies on Polynucleotides

(1) Multiple Ordered Forms of Poly(G)

We have found that there are two (or more) ordered forms of poly(G), which are quite distinct in the infrared spectra and, to a lesser extent, in their circular dichroism. We have designated the first form I, obtained by freezing and thawing a solution of the polymer. The conversion is slow for undialysed polymer, having a half time of roughly 10 hours and requiring a week or more for completion. Conversion of dialysed polymer has $t_{1/2} \cong 1$ hour and is largely complete in a day. Mg^{++} appears to increase the conversion rate of the dialysed polymer.

The I → II conversion is irreversible except by freezing and thawing the solution. Heating from I accelerates conversion to form II. Heating form II in the range 95°-100° causes little change in the infrared spectrum. The material is less than 5% melted under these conditions (e.g. 0.02 M poly(G), Na^+, no added counterion).

(2) Formation of single stranded poly(G) at high temperature

Poly(G) was converted to the $^+NEt_4$ salt by ion exchange chromatography and its infrared spectra observed as a function of temperature. A broad but cooperative melting was observed over the range ∿40°-100°, with Tm = 65°. At 98° the polymer appeared to be over 90% in the single stranded form, as

judged by characteristic ring vibrations. The melting is reversible, and the spectrum of form II reappears on cooling. This is the first time poly(G) of reasonably high MW has been obtained in single stranded form.

(3) Poly(G)·Poly(C)

Poly(G)·poly(C) in mixtures of DMSO and D_2O undergoes sharp, cooperative thermal transitions (breadth ∿10°) in a convenient temperature range: Tm 88° (50% DMSO); 72° (60% DMSO); 52° (70% DMSO). Examination of the spectra, however, shows that these are not simplex helix → coil transitions. The poly(C) is converted to its random coil form, but the poly(G) is converted to an ordered form, which undergoes no further change between the temperature of the original transition and 100°. This form of poly(G) differs from the monomer GMP or single stranded poly(G) in having ring vibations at 1588 and 1571 cm^{-1} rather than 1580 and ∿1568 cm^{-1}. A more striking difference is the low absorbance which is only about a third as intense as that of the monomer or the single stranded form. The transition is irreversible, and r(G)·r(C) is not reformed on cooling the solution. If the ordered form of poly(G) described here is four-stranded, the observed thermal transitions can be formulated as follows:

$$4 \text{ poly(G)·poly(C)} \rightarrow 4 \text{ poly(C)} + [\text{poly(G)}]_4$$

In 0.1 M Et_4N^+, r(G)·r(C) melts cooperatively with production of single stranded poly(G) and poly(C):

$$\text{poly(G)·poly(C)} \rightarrow \text{poly(G)} + \text{poly(C)}$$

The Tm is 82° under this condition, and on cooling poly(G)·poly(C) is largely reformed. There is often about 10% of G·G present after cooling, but in some runs, reformation of G·C was essentially 100%.

(4) Structure of Poly(8BrA), an All-syn Polymer

This study is continuing with a combination of experimental and theoretical methods in collaboration with Dr. Girjesh Govil of the Tata Institute, Bombay, and for the past year in the Laboratory of Chemical Physics, NIAMDD. Potential energy calculations by Dr. Govil suggest that only one of two possible hydrogen bonding schemes is likely to lead to reasonable geometry of the ribose-phosphate backbone. A new synthesis of poly(8BrA) has been carried out, producing a polymer of somewhat lower Tm and shorter chain length. We anticipate that this material will have advantages over the previous preparation for NMR investigation. (Miles, Howard and Frazier).

Structure and Mechanism of Proteases

a) Continuing the refinement of γ-chymotrypsin, 1.9 Å data were collected are are currently being used in the refinement of the structure by real space electron density fitting. It is anticipated that this refinement will soon be complete, at which point a detailed comparison will be made with the other serine proteases, trypsin, elastase and γ-chymotrypsin.

b) The acid protease from Rhizopus chinensis: A 3.0 Å map has been calculated. The backbone of the molecule has been traced for about 95% of the molecule with confidence. The binding site for inhibitors has been located in a large cleft in the molecule. Data are now being collected to a higher resolution. Several regions of low density, together with the lack of sequence data have prevented the tracing of the entire chain with complete connectivity.

Immunoglobulin Structure

1) An attempt is being made to extend the structural investigations to other immunoglobulin Fabs. Crystals have been grown for EPC 109 and TEPC 601, two mouse myeloma proteins with binding specificities for levans and galactans, respectively. Some heavy atom derivatives have been obtained for J539, another galactan specific protein, and their positions in the crystal determined.

2) The three-dimensional structure of McPC 603 has been used to understand the sequence variations in other proteins with similar binding specificities. The most probable interpretation of these data is that there is only one binding site for phosphorylcholine in these proteins. (Davies, Padlan, Navia, Silverton, Cohen, Liu).

Thermodynamics of NAD Binding to Dehydrogenases

A striking pattern of structure-function relationship has recently emerged from the X-ray crystallographic studies of several NAD-dependent dehydrogenases in spite of distinct differences in the amino acid sequences of these enzymes. The NAD-binding domains in these enzymes exhibit fundamental similarities in their structure as well as in their mode of coenzyme binding. This finding led to the suggestion that the presence of topologically related regions in enzymes of multifarious functions indicates a common evolutionary origin for these enzymes. There is also a concurrent view that the particular folding pattern observed for the NAD-binding domains is just a convenient and energetically favorable way of folding and that convergent evolution is responsible for the commonality of the super-secondary structure found in these enzymes. It is the intention of this project to link the structure-function relationship to the thermodynamics of interactions of the enzymes and NAD thereby shedding some light on the molecular nature of the structural details involved in enzyme-coenzyme interactions. It is proposed to study the binding of NAD to several dehydrogenases and evaluate the thermodynamic parameters. (Subramanian and Ross).

Kinetics and Thermodynamics of Hemoglobin S Gelation

The polymerization of hemoglobin S molecules into fibers and alignment of these fibers to form a crystalline phase (gelation) rigidifies the red cell and produces the distortion referred to as sickling. The kinetics of this process exhibit a delay period followed by a rapid, almost explosive development of the transformation. The rate (the reciprocal of the delay time) has been found to depend upon a very high power (40^{th}) of the degree of supersaturation of the solution; thus the kinetics are under thermodynamic control. We have used this supersaturation equation to predict gelation delay times

under in vivo conditions and have proposed that the probability of sickling inside capillaries is determined by the delay time of gelation. It is generally believed that, in sickle cell disease widespread sickling within the capillaries is responsible for the damage to organs of the body. The hypothesis that the clinical severity of the disease depends upon the kinetics of gelation have motivated us to further examine experimentally the basis of these predictions.

Four physical properties have been used to follow the kinetic progress curves: scanning microcalorimetry, optical birefringence, light scattering and water proton nmr linewidths. We have established that all four techniques see the onset of gelation simultaneously a result that will be useful for extension of our work to cells. We have developed a technique for determining the rate of gelation (turbidimetrically) and measuring the solubility by ultracentrifugation of the same sample in a single tube. Using this method we have confirmed the supersaturation equation which was used to predict gelation delay times under in vivo conditions and have found that the supersaturation law is also obeyed in the presence of the diverse additives; H^+, CO and urea. The finding that the supersaturation equation is obeyed in the presence of carbon monoxide strongly suggests that sickle cell hemoglobin solutions partially saturated with oxygen will also gel by the same basic mechanism we have proposed for deoxyhemoglobin S. (Ross, Hofrichter and Eaton).

Energy conversion in biology, especially muscle contraction

In previous work, we have related the mechanics of muscle contraction to the biochemical kinetics, the latter being expressed in terms of "given" rate constants. We have begun, during the current year, an attempt to go one step more deeply into the kinetics by studying the molecular theory of the rate constants themselves. One part of this work deserves special comment: it extends the range of validity of Eyring's widely used rate theory (for gas reactions) to include solution reactions and diffusion effects.

We have also investigated the connection between free energy levels of a cycling macromolecule (e.g., an enzyme) at steady-state and the kinetics of the cycle. The entire subject of steady-state kinetics and thermodynamics, and its relation to free energy transduction in biology, is being treated in a monograph that will be completed during the current year. (Hill, Plesner and Chen).

Ion transport across the nerve axon membrane

In our previous work, we were concerned with the question of how to calculate the concentration noise power spectrum of an ensemble of multi-state linear kinetic systems when the rate constants of the systems are assumed to be known. We have used a standard eigenvalue-eigenfunction method to solve the differential equations which govern the regression of the means and derived the noise power spectrum as a function of the eigenvalues and eigenfunctions of the relaxation matrix of the system. In our current work, we have obtained an equation which relates the noise spectrum matrix of the

fluctuations directly to the relaxation matrix of the means. As a result, the noise power spectrum can be calculated through matrix operations without the necessity of an eigenvalue-eigenfunction calculation. The present formalism is particularly useful in the evaluation of kinetic rate constants when the noise spectrum data of concentration fluctuations are given. Possible applications to biochemical systems have been considered. (Hill and Chen).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33000-10 LMB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Study of Functions involved in Genetic Recombination

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

PI:	Martin Gellert, Chief,	Sec. on Met. Enzymes	LMB NIAMDD
Other:	Kiyoshi Mizuuchi	Vis. Associate	LMB NIAMDD
	Mary O'Dea	Chemist	LMB NIAMDD
	Michiyo Mizuuchi	Guest Worker	LMB NIAMDD

COOPERATING UNITS (If any)

Nash, Howard	Med. Officer(Res.)	LNC NIMH

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Metabolic Enzymes

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2	1.5

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

A novel assay has been developed for studying this site-specific recombination in vitro. The substrate (developed by H. Nash) is a λ DNA containing two sets of attachment sites; recombination excises the fragment between the sites. The assay looks at the sizes of restriction fragments produced by the Eco RI enzyme. The recombined products have different fragment sizes, as measured by electrophoresis in agarose gels, from the starting DNA.

Using this assay, the following results have been obtained: 1) Covalently circular DNA is the only effective substrate. Linear DNA is inactive; nicked circular DNA works only if it can be sealed by DNA ligase in the reaction mixture. 2) Both recombination products the excised fragment and the remaining λ DNA) are recovered in equal yield. 3) Both recombination products are recovered largely in the form of covalent circles, even when DNA ligase in the cell extracts is inhibited by NMN. This raises the possibility of a ligase-independent sealing step as a coupled part of the recombination process. 4) If the products are not treated with restriction enzyme, most of the excised fragment is recovered as circles interlocked (catenated) with the circular λ DNA product. This result implies that recombination occurs while the substrate DNA is supercoiled.

PHS-6040
(12-75)

Project Description:

1) Studies on integrative recombination of phage λ in a cell-free system (K. Mizuuchi, H. Nash, M. Gellert, M. O'Dea and M. Mizuuchi)

a) A novel assay has been developed for studying this site-specific recombination in vitro. The substrate (developed by H. Nash) is a λ DNA containing two sets of attachment sites; recombination excises the fragment between the sites. The assay looks at the sizes of restriction fragments produced by the Eco R1 enzyme. The recombined products have different fragment sizes, as measured by electrophoresis in agarose gels, from the starting DNA.

Using this assay, the following results have been obtained:

1) Covalently circular DNA is the only effective substrate. Linear DNA is inactive; nicked circular DNA works only if it can be sealed by DNA ligase in the reaction mixture.

2) Both recombination products (the excised fragment and the remaining λ DNA) are recovered in equal yield.

3) Both recombination products are recovered largely in the form of covalent circles, even when DNA ligase in the cell extracts is inhibited by NMN. This raises the possibility of a ligase-independent sealing step as a coupled part of the recombination process.

4) If the products are not treated with restriction enzyme, most of the excised fragment is recovered as circles interlocked (catenated) with the circular λ DNA product. This result implies that recombination occurs while the substrate DNA is supercoiled.

b) A search is being continued for host mutants in which λ integration is defective. Several candidate mutants have been isolated and are now being tested.

2) Assays for general genetic recombination in a cell-free system (M. Gellert, M. O'Dea).

Two assays are being developed for studying this process. Both use a λ phage genome carrying a duplicated region which can be excised by general recombination. In one assay, the product is recognized by a changed pattern of restriction fragments (as in the assay for site-specific recombination described above); in the other, a positive selection for reduced DNA size is used. There are technical difficulties in both methods but these do not seem insoluble.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33001-07 LMB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Protein Synthesis in Mammals: Mechanisms and Metabolic Controls

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

PI:	Elizabeth S. Maxwell	Research Chemist	LMB NIAMDD
OTHER:	G. Stanley Cox	Staff Fellow	LMB NIAMDD
	Miin-Rong Tsai	NIH Postdoc. fellow	LMB NIAMDD
	Elizabeth A. Robinson	Research Chemist	LCB NCI
	Bruce D. Weintraub	Sen. Investigator	CEB NIAMDD
	Saul W. Rosen	Sen. Investigator	CEB NIAMDD

COOPERATING UNITS (if any)

LCB, NCI
CEB, NIAMDD

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Metabolic Enzymes

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1.0

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

The long range objective of this project is to understand at the molecular level how proteins are synthesized in mammals and how the process of protein synthesis is controlled at the levels of translation and transcription. The topics of current interest are: 1) the general protein chemistry of eukaryotic polypeptide chain elongation factors 2) the identificaiton of active sites and sites of metabolic control and the determination of amino acid sequences of such sites 3) identification of an unusual amino acid at the site of ADP ribosylation and inactivation of elongation factor 2 with NAD^+ and diphtheria toxin 4) the properties of biologically active messenger RNA for human placental polypeptide hormones 5) translation of eukaryotic mRNA in a cell-free system from wheat germ.

PHS-6040
(12-75)

Project Description:

The overall long range objective of this project is to understand at the molecular level how proteins are synthesized in eukaryotes and how protein synthesis is controlled at the levels of transcription and translation of messenger RNA.

The project will be described in two sections. The first has to do with the process of polypeptide chain elongation and with some properties of the soluble protein factors required. The second is concerned with biologically active mRNA from human placenta and with its translation in cell-free systems.

Polypeptide chain elongation:

The purpose of these investigations is to gain an understanding of the catalytic mechanisms and metabolic controls of polypeptide chain elongation. Our current approach is to identify active sites and potential regulatory sites on the essential protein factors and to determine the amino acid sequences around these sites.

This year, using a new combination of methods, we have compared the tryptic peptide maps of eukaryotic elongation factor 2 (EF-2) from rat liver and from rabbit reticulocytes with that of the analogous prokaryotic factor (EF-G) from E. coli. The differences between the maps of either EF-2 and that of EF-G are extensive. All three factors require at least one free sulfydryl group for activity. We have labeled the cysteines of EG-G and of rat liver EF-2 with ^{14}C ethyleneimine in order to identify a potentially conserved active site. The labeled peptides are now being purified prior to determination of their amino acid sequence.

All preparations of EF-2 tested to date are ADP-ribosylated in the presence of diphtheria toxin and NAD^+ and are thereby inactivated. This reaction is believed to be responsible for the in vivo effects of the toxin. EF-G is neither inactivated nor ADP-ribosylated by toxin and NAD^+. We have previously determined the amino acid sequence at this potentially regulatory site on the protein factor around the covalently attached ADP-ribose. The ADP-ribose is linked to an unusual amino acid (X) which has not been reported previously in proteins. Free X has been isolated following acid hydrolysis of the tryptic peptide and X-ribose was isolated following enzymatic degradation, but we have not yet identified the unknown residue. This year we have purified a tryptic peptide containing X from native EF-2 which had not been ADP-ribosylated. This peptide has the same amino acid composition as that containing X-ribose. We are currently attempting to isolate sufficient free X by mild or enzymatic degradation to study the compound by gas chromatography-mass spectrometry and perhaps by nuclear magnetic resonance. A knowledge of the structure of X may provide insight into the catalytic mechanism and control of translocation, the step in polypeptide chain elongation catalyzed by EF-2. As anticipated EF-G does not contain X.

Biologically active Messenger RNA from Human Placenta:

The long range purpose of this part of the project is to understand regulation of human placental polypeptide hormone synthesis at the levels of transcription and translation, and to examine the mechanism by which one of these proteins (the α subunit of human chorionic gonadatropin) is produced ectopically by several cell lines derived from human carcinomas.

Our current approach is to examine the biological activity of placental mRNA in cell-free systems for protein synthesis. The poly(A) containing fraction of total placental RNA directs the synthesis of two polypeptides precipitable by antiserum directed against human placental lactogen (hPL). Electrophoresis on SDS polyacrylamide gels reveals that one product co-migrates with authentic hPL, while the other migrates slightly slower and has an apparent molecular weight about 3000 units larger. We are presently investigating the possible precursor-product relationship of the two polypeptides. We anticipate extending this work to include translations of mRNA isolated from cells in culture derived from human carcinomas.

Publications:

Cox, G. S., Weintraub, B. D., Rosen, S. W. and Maxwell, E. S.: Properties of Biologically Active Messenger RNA from Human Placenta. Cell-Free synthesis of two immunoreactive forms of placental lactogen. J. of Biol. Chem. 251: 1723-1730, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33002-03 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structural Studies on Mouse Myeloma Immunoglobulins

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

PI:	Elizabeth Robinson	Research Chemist	Formerly LMB NIAMDD
	Ettore Appella	Med. Officer	LCB NCI

COOPERATING UNITS (if any)

LCB, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Metabolic Enzymes

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0		

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

Project terminated in Fiscal year 1975.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33003-01 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

DNA replication in vitro catalyzed by purified E. coli proteins

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

PI: Sue Wickner Staff Fellow LMB NIAMDD

COOPERATING UNITS (if any)
Dr. J. Hurwitz, Albert Einstein College of Medicine, Bronx, N.Y.
Dr. C. Raetz, LBP NIAMDD

LAB/BRANCH
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SECTION
Section on Metabolic Enzymes

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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

Three pathways have been identified by which single-stranded circular phage DNAs are converted to duplex DNA in reactions catalyzed by purified proteins isolated from uninfected E. coli: (1) DNA synthesis of fd or M13 DNA requires RNA polymerase, DNA binding protein, DNA elongation factors I and III, dnaZ gene product, and DNA polymerase III; (2) Synthesis of ST-1 or G4 DNA requires dnaG and dnaZ gene products, DNA binding protein, DNA elongation factors I and III and DNA polymerase III; and, (3) Synthesis of ØX174 DNA requires dnaB, dnaC, dnaG, and dnaZ gene products, DNA binding protein, DNA elongation factors I and III, DNA polymerase III and replication factors X, Y, and Z. The functions of these purified proteins have been studied: (1) The dnaB gene product has ribonucleoside triphosphatase activity which is stimulated by single-stranded DNA; (2) dnaB and dnaC gene products interact physically and functionally in vitro; (3) Replication factor Y has ØX174 DNA-dependent ATPase activity; and, (4) dnaZ gene product and elongation factors I and III function with either DNA polymerase III or II in the elongation of single-stranded DNA primed with DNA or RNA.

PHS-6040
(12-75)

Project Description:

Background

The purpose of my research has been to study the proteins involved in Escherichia coli DNA replication and to elucidate basic biochemical mechanisms involved in this central process of heredity and cell growth. Studies of replication in E. coli have been made possible by the isolation by others of mutants defective in DNA synthesis. Genes whose functions are required for chromosome replication have been designated dnaA, dnaB, dnaC(D), dnaE, dnaG, dnaI, dnaP, dnaZ, lig and polA. Some of these proteins are also required for the conversion of fd, ST-1 and ØX174 single-stranded phage DNAs to duplex DNA in vitro. Thus stimulation of DNA synthesis in inactive crude extracts of dna mutants by fractions from wild-type cells has provided complementation assays for purification of some of the dna gene products. These proteins are not sufficient to catalyze DNA synthesis dependent of fd, ST-1 or ØX174 DNA. Other proteins required for these reactions have been isolated and the reaction requirements are as follows:

	fd	ST-1	ØX174
Specific DNA, dNTPs, Mg^{+2}	+	+	+
ATP	+	-	+
UTP, GTP, CTP	+	-	-
RNA polymerase	+	-	-
dnaG gene product	-	+	+
dnaB, dnaC(D) gene products, factors X, Y, Z	-	-	+
DNA binding protein	+	+	+
Elongation factors I and III	+	+	+
dnaZ gene product	+	+	+
DNA polymerase III (dnaE)	+	+	+

Recent Results:

A. Involvement of dnaZ gene product in DNA elongation.

The dnaZ gene product is required in all three of these DNA synthesizing systems. Using this requirement it has been purified about 5000-fold. It functions in the elongation of RNA or DNA-primed single-stranded DNA catalyzed by DNA polymerase III in conjunction with two other E. coli proteins referred to as DNA elongation factors I and III, in a reaction requiring ATP or dATP. It also functions in similar reactions catalyzed by DNA polymerase II in combination with E. coli DNA binding protein and DNA elongation factors I and III. Elongation factors I and III have been purified by their requirement in this reaction but have not been identified genetically yet.

B. DNA-dependent ATPase activity of replication factor Y.

The association of DNA-dependent ATPase activity with factor Y was shown in the following ways: (1) the 2 activities copurified over 2 consecutive purification steps with a constant ratio of factor Y to ATPase; (2) they comigrated on native polyacrylamide gel electrophoresis; (3) both activities were heat inactivated at the same rate; and (4) both showed identical patterns of N-ethylmaleimide sensitivity. Factor Y hydrolyzes ATP or dATP producing P_i and ADP or dADP; other NTPs and dNTPs are not hydrolyzed. The reaction is stimulated 50 to 100 fold by ØX174 DNA. Other single-stranded DNA, double-stranded DNA and RNA are less effective in stimulating ATPase activity (1 to 5 fold). It is noteworthy that factor Y is required for ØX174 DNA synthesis and not for fd or ST-1 DNA synthesis and similarly the ATPase is stimulated 20 fold more effectively by ØX174 DNA than by fd or ST-1 DNA.

C. Resolution of ØX174 DNA system into 2 steps.

Using the reconstituted ØX174 DNA replicating system it is now possible to further dissect DNA replication into its partial reactions. So far the reaction has been resolved into two steps. Step one requires dna B and dna C(D) gene products in addition to DNA binding protein and replication factors X, Y and Z, ATP and ØX174 DNA and involves reactions prior to dNMP incorporation; dna G and dna Z gene products, DNA polymerase III and DNA elongation factors I and III are not required during step 1 but are required for dNMP incorporation during the second step following the addition of dNTPs. Incubation of the components required for step 1 followed by gel filtration resulted in the isolation of a complex associated with ØX174 DNA in the excluded volume. The addition of the components required for step 2 to this complex resulted in dNMP incorporation. Analysis of the complex isolated after gel filtration indicated that 25 to 50% of the dna B gene product recovered was associated with the ØX174 DNAin the excluded volume; the rest was partially included in the gel. In contrast, <5% of the dna C(D) gene product recovered was associated with the DNA; all detectable dnaC activity was associated with dna B in the partially included volume or free in the fully included volume. Thus it is possible that the complex of dna B and dna C(D) gene products participates in a reaction resulting in the transfer of dna B to ØX174 DNA and the concomitant release of dna C(D) gene product.

D. Over production of dna gene products by E. coli strains carrying hybrid Col El plasmids.

To facilitate purification of dna gene products, a bank of 2000 E. coli strains carrying Col El plasmids into which small random segments of the E. coli chromosome are inserted (Clarke, L., and Carbon, J. (1975) Proc. Nat Acad. Sci., 72, 4361) was screened for those hybrids transferring thermoresistance to dna temperature sensitive mutants. One or more was found to transfer dnaB, dnaC, dnaE and dnaZ; none was found to transfer dnaG. These donor strains each overproduce by 3- to 10-fold to product of the gene whose defect is corrected, except in the case of dnaE. The strain donating dnaE does not overproduce DNA polymerase III.

Proposed course:

I am interested in studying biochemical mechanisms involved in DNA synthesis using purified proteins and defined DNA templates.

Publications:

Wickner, S. and Hurwitz, J.: In vitro Synthesis of DNA. In Goulian, M. M., Hanawalt, P. C. and Fox, C. F. (Eds.): DNA synthesis and its Regulation. Menlo Park, Calif., W. A. Benjamin, Inc., 1975, pp. 227-238.

Wickner, S. and Hurwitz, J.: Association of ØX174 DNA-dependent ATPase activity with an E. coli protein, replication factor Y, required for in vitro synthesis of ØX174 DNA. Proc. Nat. Acad. Sci., U.S.A. 72: 3342, 1975.

Wickner, S. and Hurwitz, J.: Involvement of E. coli dnaZ gene product in DNA elongation. Proc. Nat. Acad. Sci., U.S.A. 73: 1053-1057, 1976.

Wickner, S., Wickner, R. and Raetz, C.: Overproduction of dna gene products by Escherichia coli strains carrying hybrid Col E1 plasmids. Biochem. Biophys. Res. Commun. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33051-04 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Mammalian Enzymes Involved in Repair or Replication of DNA

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

PI:	S. B. Zimmerman	Research Chemist	LMB NIAMDD
OTHER:	C. J. Levin	Chemist	LMB NIAMDD

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	.5	1

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

The aim of this project is to identify and characterize enzymes involved in the repair and replication of DNA in higher organisms. Currently we are
1) Examining the number and characteristics of DNA ligases in rat liver,
2) Characterizing a DNA kinase of unusual specificity also from that source.

PHS-6040
(12-75)

Project Description:

Objectives: To isolate and characterize enzymes from mammalian sources which act on DNA, such as DNA kinase and DNA ligase, in order to attempt to understand more fully the processes of DNA repair and replication.

Methods: Standard techniques of biochemistry.

Major Findings: Several percent of the total DNA of eukaryotic cells may be contained within mitochondria. This mitochondrial DNA is often in a closed circular form, implying the action of a DNA ligase-like activity for at least one step in its replication. We have found an ATP-dependent DNA ligase in mitochondria isolated from rat liver. This activity is released by osmotic and other treatments in the manner expected for an intramitochondrial localization. The properties of the partially purified enzyme are similar to those of the nuclear DNA ligase from rat liver but differ from some of those reported for a "soluble" cytoplasmic ligase.

A DNA kinase of unique specificity has been identified in rat liver. The kinase has been partially purified from rat liver nuclei by a procedure which also yields DNA ligase. The kinase uses ATP to phosphorylate specifically the 5'-hydroxyl termini of oligodeoxynucleotides and of single- or double-stranded DNA, yielding 5'-phosphate termini and ADP. The kinase is inactive on RNA, or on oligodeoxynucleotides of chain length less than approximately 10 to 12 residues. The kinase requires a divalent cation (Mg^{++}, Mn^{++}, Co^{++}, Zn^{++}, Ni^{++}, or Ca^{++}) for activity and has an acidic pH optimum. It is inhibited by a variety of nucleotides as well as by very low levels of inorganic and organic sulfate compounds and sulfate analogues. The molecular weight of the kinase is estimated to be 8×10^4 from gel filtration.

Significance to Bio-Medical Research and the Program of the Institute: The ligase and kinase, as well as other enzymes, are thought to function in repair and replication of DNA. Damaged regions in DNA have been implicated in a variety of disease states as well as in aging and differentiation. Enumeration and characterization of the enzymes which carry out repair and replication will hopefully lend insight into the mechanisms of these processes and the aberrations in them which occur in the various diseases states.

Proposed Course: A unique cytoplasmic DNA ligase recently has been identified in rat liver by Tsukada and Ichimura (FEBS Lett. 54, 217 (1975)). We have confirmed their result and are purifying this enzyme in an attempt to see its relation to the other known ligases of this tissue which we have been studying for several years.

Characterization of the DNA kinase will be continued. Early results indicate the activity to be inhibited by many normally occurring metabolites, and hence readily subject to control. Such control has the potential for controlling DNA degradation and ligation, since the kinase can change the

nature of the termini of DNA.

Publications:

Levin, C. J. and Zimmerman, S. B.: A Deoxyribonucleic Acid Kinase from Nuclei of Rat Liver: Purification and Properties. J. Biol. Chem. 251: 1767-1774, 1976.

Levin, C. J. and Zimmerman, S. B.: A DNA Ligase from Mitochondria of Rat Liver. Biochem. Biophys. Res. Communs. 69: 514-520, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33052-03 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
X-Ray Diffraction Studies on Polyguanylic Acid

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

PI:	S. B. Zimmerman	Research Chemist	LMB NIAMDD
OTHER:	D. R. Davies	Chief, Mol. Structure	LMB NIAMDD
	G. H. Cohen	Research Chemist	LMB NIAMDD

COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

This project was completed in Fiscal year 1975.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 33053-04 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Kinetics and Thermodynamics of Hemoglobin S Gelation

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

PI:	Philip D. Ross	Research Chemist	LMB NIAMDD
OTHER:	James Hofrichter	Staff Fellow	LCP NIAMDD
	William A. Eaton	Senior Surgeon	LCP NIAMDD

COOPERATING UNITS (If any)

NONE

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	2.7	

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

The polymerization of hemoglobin S molecules into fibers and alignment of these fibers to form a crystalline phase (gelation) rigidifies the red cell and produces the distortion referred to as sickling. In addition to its importance in disease this polymerization-crystallization phenomenon provides an excellent example of a macromolecular assembly process.

The aim of this project is to gain a basic physical-chemical understanding of the sickling phenomenon and to interpret the behavior of potentially therapeutic additives in terms of their effects upon the kinetics and thermodynamics of the gelation of hemoglobin S.

PHS-6040
(12-75)

Project Description:

Objectives: To gain a basic physical-chemical understanding of the sickling phenomenon and to interpret the behavior of potentially therapeutic additives in terms of their effects upon the kinetics and thermodynamics of gelation.

Methods: Differential scanning and isothermal calorimetry. Optical birefringence and light scattering. Ultracentrifugation and measurement of water proton line widths.

Major Findings: The previously proposed empirical equation relating the rate of gelation to the degree of supersaturation of the hemoglobin S solution has been confirmed and found also to be applicable in the presence of the diverse additives; H^+, CO and urea. These results strongly support our methods of predicting gelation delay times under in vivo conditions. The results with carbon monoxide strongly indicate that sickle cell hemoglobin solutions saturated with oxygen will undergo gelation by the same basic mechanism we have already observed.

We have established that the four different physical techniques of microcalorimetry, optical birefringence, light scattering and water proton nmr linewidths see the onset of gelation simultaneously. This result coupled with our calibration of these signals by solubility studies should provide a basis of interpreting physical measurements on cells.

A detailed study of the solubility, enthalpy and heat capacity changes associated with gelation has been completed.

Application of multicomponent thermodynamic theory in addition to accounting for some of the details of our thermodynamic results also provides a basis for the interpretation of experiments on the gelation behavior of mixtures containing different hemoglobin species and experiments containing mixtures of partially liganded hemoglobin S. A simple physical picture of the polymerization of hemoglobin S has been developed from which the supersaturation law is derived, thus demonstrating theoretically the intimate connection between the thermodynamics and kinetics of this system that is observed experimentally.

Significance to Biomedical Research and the Program of the Institute: The results and implications of our kinetic study bring a new point of view toward understanding the pathophysiology of sickle cell disease.

The mechanism and kinetic theory developed in this study may be applicable to other macromolecular assembly processes.

The methods so far developed will be useful in examination and interpretation of the effects of potential therapeutic agents upon the sickling process.

Proposed Course of Project: The fundamental physical-chemical experiments and theoretical work will be continued. The effects of additives will be studied. These studies will be extended to the sickling process in erythrocytes.

Publications:

Eaton, W. A., Hofrichter, J. and Ross, P. D.: Comparison of Sickle Cell Hemoglobin Gelation Kinetics Measured by NMR and Optical Methods. Biochem. and Biophys. Res. Communs. 69: 538-547, 1976.

Eaton, W. A., Hofrichter, J. and Ross, P. D.: Delay Time of Gelation: A Possible Determinant of Clinical Severity in Sickle Cell Disease. Blood 47: 621-627, 1976.

Hofrichter, J., Ross, P. D. and Eaton, W. A.: A Thermodynamic and Kinetic Description of the Gelation of Deoxyhemoglobin S. Proceedings of the Symp. on Molecular and Cellular Aspects of Sickle Cell Disease. Dallas, Texas, 1975 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33054-01 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Structural Studies on Polynucleotides

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

PI: S. B. Zimmerman Research Chemist LMB NIAMDD

COOPERATING UNITS (If any)
NONE

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	0

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

The aim of this project is to obtain structural information on polynucleotides and to provide insights into the factors which control structure, principally through X-ray fiber diffraction and model building studies. Current areas include 1) the structure of the polyU-spermine complex, 2) Prediction of structural parameters from observed helical parameters, 3) The structure of a polynucleotide-like helical complex adopted by a monomer (G-5'-P).

PHS-6040
(12-75)

Project Description:

Objectives: Obtain structural information on polynucleotides and to provide insights into the factors which control structure.

Methods: Standard techniques of X-ray diffraction and biochemistry; computerized molecular model building.

Major Findings: The poly U-spermine complex has been studied by X-ray fiber diffraction techniques. The X-ray pattern is generally similar to that of A RNA or the A form of DNA, suggesting a double-helical structure with strands of opposite polarity. The number of residues per turn of the helix is not well-defined by the diffraction data; there are probably from 9 to 11 residues/turn.

G-5'-P crystallizes from neutral solutions of NaCl in bundles of hair-like crystals. Dried bundles of these crystals give a fiber-like X-ray diffraction pattern which indicates that the monomers are arranged similarly to the arrangement of residues in poly G, and hence represent a unique helical aggregate.

A geometrical approach to helical polynucleotide structures allows prediction of a number of structural parameters from very limited input data. For example, the radial position of the backbone may be predicted from the observed helical parameters. This approach may also be used to evaluate the tilt and radial position of the bases for a given H-bonded base arrangement.

Significance to Bio-Medical Research and the Program of the Institute: Polynucleotides are crucial in controlling cells in both normal and diseased states. Understanding of polynucleotide structure will hopefully be of aid in understanding and interacting with cells in such diseased states.

Proposed Course: Detailed model building and chemical studies are in progress to determine the structure and stoichiometry of the poly U-spermine complex. The limits to the geometrical approach to structure prediction are being systematically examined.

Publications:

Zimmerman, S. B.: An "Acid" Structure for Polyriboguanylic Acid Observed by X-ray Diffraction. Biopolymers 14: 889-890, 1975.

Zimmerman, S. B.: The Polyuridylic Acid Complex with Polyamines: An X-ray Fiber Diffraction Observation. J. Mol. Biol. 101: 563-565, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33055-01 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Thermodynamics of NAD Binding to Dehydrogenases

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

PI:	S. Subramanian	Visiting Associate	LMB NIAMDD
OTHER:	P. D. Ross	Research Chemist	LMB NIAMDD

COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	0

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

A striking pattern of structure-function relationship has recently emerged from the X-ray crystallographic studies of several NAD-dependent dehydrogenases in spite of distinct differences in the amino acid sequences of these enzymes. The NAD-binding domains in these enzymes exhibit fundamental similarities in their structure as well as in their mode of coenzyme binding. This finding led to the suggestion that the presence of topologically related regions in enzymes of multifarious functions indicates a common evolutionary origin for these enzymes. There is also a concurrent view that the particular folding pattern observed for the NAD-binding domains is just a convenient and energetically favorable way of folding and that convergent evolution is responsible for the commonality of the supersecondary structure found in these enzymes. It is the intention of this project to link the structure-function relationship to thermodynamics of interactions of the enzymes and NAD thereby shedding some light on the molecular nature of the structural details involved in enzyme-coenzyme interactions. It is proposed to study the binding of NAD to several dehydrogenases and evaluate the thermodynamic parameters.

PHS-6040
(12-75)

Project Description:

Objectives: (1) To obtain thermodynamic parameters for the binding of NAD to several dehydrogenase. (2) To examine the thermodynamic data in relation to structural and functional aspects of the enzymes and attempt to interpret the molecular basis of enzyme-coenzyme interactions.

Methods: Batch and Flow Microcalorimetry.

Major Findings: Thermodynamic data for NAD binding have been obtained for three different dehydrogenases. They suggest some similarities besides some interesting differences.

Significance to Biomedical Research and the Program of the Institute: This project is designed to enable a better understanding of the structure-function relationship of enzymes through the thermodynamic investigation of enzyme-coenzyme interactions using microcalorimetry as a tool. The basic knowledge gained thereby could pave the way for understanding the mechanistic aspects of the glycolytic enzymes.

Proposed Course: To pursue the study along the same lines for more dehydrogenases and investigate the relationship between molecular structure and thermodynamics.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 34001-11 LMB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Chromatin Structure and Function

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

PI:	G. Felsenfeld	Chief, Sec. on Phys. Chem.	LMB NIAMDD
OTHER:	R.D. Camerini-Otero	Research Associate	LMB NIAMDD
	W. Melchior, Jr.	Senior Staff Fellow	LMB NIAMDD
	J. McGhee	Guest Worker	LMB NIAMDD
	G. Sandeen	Chemist	LMB NIAMDD
	R. Simon	Research Associate	LMB NIAMDD
	B. Sollner-Webb	Staff Fellow	LMB NIAMDD
	P. Williamson	Staff Fellow	LMB NIAMDD
	M. Zasloff	Research Associate	LMB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Physical Chemistry Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.4	7.4	1

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

The broad purpose of this project is to understand the way in which chromatin, the DNA-protein complex in the nucleus of higher organisms, operates to establish and maintain regulated cell function. Investigations are in progress to understand chromatin structure, i.e. the specific organization of proteins and DNA within the complex, and the relationship of this structure to biological functions. The biological function of present interest is control of gene expression: the proteins of chromatin serve to block certain gene sequences from being transcribed into messenger RNA. This is believed to form the chemical basis of cell differentiation. One test system being investigated is globin messenger RNA synthesis in chromatin from avian reticulocytes. The goal is to isolate the biochemical factors in this chromatin responsible for maintaining globin message synthesis, and to understand how they affect chromatin structure to carry out their function. Other related studies are concerned with the folding of DNA by chromatin proteins, the physical arrangement of proteins on the globin gene, and the effects of these proteins on the transcription process.

PHS-6040
(12-75)

Project Description:

Objectives: The object of this project is to determine the structure of chromatin and the relationship of this structure to its biological role in cell differentiation.

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. DNA-RNA hybridization methods are used to detect presence of specific polynucleotide sequences. Gel electrophoresis methods are used for measuring DNA and protein size, for examining nucleoprotein preparations, and for detecting chemically cross-linked proteins within chromatin.

Major Findings: We have continued our study of chromatin structure, using a variety of chemical probes of both DNA and protein conformation. We find that digestion of chromatin with the enzyme staphylococcal nuclease results in the appearance of a series of discrete double stranded DNA fragments that are approximately multiples of ten nucleotide pairs in length. Digestion with pancreatic DNase (DNase I) or spleen acid DNase (DNase II) leads to production of single stranded DNA fragments (following denaturation) of discrete sizes that are also multiples of ten nucleotides in length. We have used the appearance of these fragment patterns on electrophoretic gels as a diagnostic of the organization of DNA structure in a chromatin-like fashion, and asked which histones (the basic proteins that comprise the bulk of chromatin protein) are responsible for generating this organization. Virtually all possible combinations of the four histone species known to be involved in DNA organization were reconstituted in complexes with DNA, and subject to digestion by the nuclease probes. We find that regeneration of chromatin-like structure is absolutely dependent upon the presence of the two arginine-rich histones, H3 and H4, and that the presence of these two histones is sufficient to generate a large part of the banded DNA digestion pattern on electrophoretic gels. Examination of the kinetics of digestion confirms that H3/H4 are capable of transiently stabilizing DNA segments approximately the length of the nucleosome, the fundamental subunit of chromatin structure. Direct measurement of the interaction of H3/H4 mixtures with DNA 140 base pairs in length (the size of the nucleosome core) indicates that a tetramer of two H3 and two H4 molecules are bound to each DNA molecule.

Further experiments with the proteolytic enzymes trypsin and chymotrypsin show that H3 and H4 are required in histone-DNA reconstitutes to generate structures in which the histones are partially protected from proteolytic attack. These results confirm the central role of histones H3 and H4 in organizing chromatin structure.

We are also continuing our studies of the relationship between chromatin structure and function. In earlier published studies, we demonstrated that chromatin isolated from avian reticulocytes served as a template for transcription with E. coli RNA polymerase to produce RNA enriched in globin messenger RNA sequences, while chromatin from other tissues did not. We have continued this work, introducing a much more sensitive method for the detection of newly synthesized RNA which depends upon the use of mercury-substituted UTP in the in vitro synthesis mixture. The newly synthesized RNA, which now contains covalently bound mercury atoms, is easily purified from any endogenous message that may contaminate the chromatin. Using radioactive globin cDNA as a probe in hybridization experiments, it is then easy to measure net globin RNA synthesis. We are able to confirm our earlier results quantitatively: about one part in 10^4 of the in vitro transcript from chromatin isolated from immature duck red blood cells is globin RNA. We have also repeated and extended our early experiments showing that reconstituted chromatin from these sources retains this transcriptional specificity. The new techniques are more efficient and sensitive than earlier ones, and open the way to large scale efforts at purification of control factors in globin message synthesis.

Significance to Bio-medical Research and the Program of the Institute:

Chromatin is the nucleoprotein complex that appears to contain the chemical information for cellular differentiation. Understanding of the organization of chromatin proteins on DNA and the way in which they affect transcription and other DNA functions in higher organisms will lead to an understanding of how cells differentiate, and how they maintain this differentiated state, in which only a relatively small fraction of the total gene population of the cell is expressed. This is a central problem of cell biology.

Proposed Course of Project: We will continue to study the role of the arginine-rich histones in stabilizing chromatin structure, using DNA fragments of well-defined size. We are also undertaking the purification of factors responsible for maintaining globin gene transcription in avian reticulocyte chromatin, using the improved assay we have developed.

Publications:

Axel, R., Cedar, H. and Felsenfeld, G.: The Structure of the Globin Genes in Chromatin. Biochemistry 14: 2489-2495, 1975.

Sollner-Webb, B. and Felsenfeld, G.: A Comparison of the Digestion of Nuclei and Chromatin by Staphylococcal Nuclease. Biochemistry 14: 2915-2920, 1975.

Sollner-Webb, B. and Felsenfeld, G.: Protein Interaction with DNA in Chromatin. In Stein, G. (Ed.): Chromosomal Proteins and Their Role in the Regulation of Gene Expression. New York, Academic Press, Inc., 1975, pp. 213-226.

Felsenfeld, G., Sollner-Webb, B. and Camerini-Otero, R.D.: Organization of Chromatin Proteins. In Tso', P.O.P. (Ed.): Molecular Biology of the Mammalian Genetic Apparatus - Its Relationship to Cancer, Aging and Medical Genetics, Part A, North Holland, Associated Scientific Publishers, in press.

Camerini-Otero, R.D., Sollner-Webb, B. and Felsenfeld, G.: The Organization of Histones and DNA in Chromatin: Evidence for an Arginine-Rich Histone Kernel. Cell, in press.

Felsenfeld, G., Sollner-Webb, B., Camerini-Otero, R.D. and Melchior, W. Jr.: Organization of Proteins in Chromatin. Symposium on the Molecular Biology of Hormone Action, Society for Developmental Biology, in press.

Felsenfeld, G., Camerini-Otero, R.D. and Sollner-Webb, B.: The Structure of Chromatin and Its Reconstruction. In Nierlich, D.P. and Rutter, W. (Eds.): Molecular Mechanisms in the Control of Gene Expression, California, W.A. Benjamin, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 34002-12 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
X-ray Diffraction Investigation of Proteolytic Enzymes

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

PI:	D.R. Davies	Chief, Sec. on Mol. Struc.	LMB NIAMDD
OTHER:	E. Subramanian	Visiting Associate	LMB NIAMDD
	G. Cohen	Research Chemist	LMB NIAMDD
	E. Silverton	Research Chemist	LMB NIAMDD
	M. Liu	Visiting Associate	LMB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3-1/4	3-1/4	0

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

Continuing the refinement of γ-chymotrypsin, 1.9 Å data have been collected and are currently being used in the refinement of the structure by real space electron density fitting. It is anticipated that this refinement will soon be complete, at which point a detailed comparison will be made with the other serine proteases, trypsin, elastase and γ-chymotrypsin.

The acid protease from Rhizopus chinensis: A 3.0 Å map has been calculated. The backbone of the molecule has been traced for about 95% of the molecule with confidence. The binding site for inhibitors has been located in a large cleft in the molecule. Data are now being collected to a higher resolution. Several regions of low density, together with the lack of sequence data have prevented the tracing of the entire chain with complete connectivity.

PHS-6040
(12-75)

Project Description:

Objectives: The determination of the three-dimensional structure of proteolytic enzymes, in particular, the structure of γ-chymotrypsin and of the acid protease from Rhizopus chinensis.

Methods Employed: X-ray diffraction analysis.

Major Findings: 1. The refinement of tosylchymotrypsin at 2.7 Å is now complete. The refinement of native γ-chymotrypsin to 1.9 Å is in progress, by means of repeated Diamond type refinements of $(2F_o-F_c)$ syntheses.

2. A 3.0 Å map of the acid protease from Rhizopus chinensis has been calculated and the polypeptide backbone followed for most of the molecule. The molecule is bi-lobal with a large cleft in which inhibitors have been observed to bind. The polypeptide backbone exists mainly in the form of antiparallel pleated sheets. Comparison with the 3.0 Å map of an acid protease from Endothia Parasitica (Blundell & Jenkins, unpublished) has shown the two structures to be very closely similar, if not identical.

Proposed Course of Project: to calculate a 2.5 Å map and construct a model of the acid protease. 2.3 Å native data are already available for refinement.

Significance to Bio-medical Research and the Program of the Institute:

Although the general mechanism of action of the serine proteases is fairly well understood, there is still some disagreement concerning the precise details. The refinement of γ-chymotrypsin at high resolution and different pH's should help resolve the problem.

The acid protease is one of a large class of enzymes that include pepsin, rennin and the cathepsins. It has sequence homology with other members of the class and the elucidation of its structure should ultimately reveal the mechanism of action of these proteins.

Publications:

Subramanian, E., Swan, I.D.A. and Davies, D.R.: The Crystal Structure at 5.5 Å Resolution of an Acid-Protease from Rhizopus Chinensis. Biochem. and Biophys. Res. Comm. 68: 875-880, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 34003-08 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Crystal Structure Investigation of Antigen Antibody Interaction

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

PI:	D.R. Davies	Chief, Sec. on Mol. Struc.	LMB NIAMDD
OTHER:	E. Padlan	Visiting Scientist	LMB NIAMDD
	E. Silverton	Research Chemist	LMB NIAMDD
	M. Navia	Staff Fellow	LMB NIAMDD

COOPERATING UNITS (if any)

M. Potter	Biologist	LCB NCI
S. Rudikoff	Microbiologist	LCB NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

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

We are extending the structural investigations of McPC 603 to other immunoglobulin Fabs. Crystals have been grown for EPC 109 and TEPC 601, two mouse myeloma proteins with binding specificities for levans and galactans, respectively. Some heavy atom derivatives have been obtained for J539, another galactan specific protein, and their positions in the crystal determined.

The three-dimensional structure of McPC 603 has been used as a basis for understanding the sequence variations in other proteins with similar binding specificities. The most probable interpretation of these data is that there is only one binding site for phosphorylcholine in these proteins.

A model of BS5, an anti-type III polysaccharide antibody from the rabbit, has been constructed. The model has a large cleft in which it is possible to bind a hexasaccharide molecule.

PHS-6040
(12-75)

Project Description:

Objectives: A comparative investigation of the crystal structure of the Fab fragment of immunoglobulin molecules that bind strongly to several different 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. Model building. Computer comparisons of structure.

Major Findings: 1. During the last year, a major attempt has been made to obtain crystals of new immunoglobulin Fabs. Two proteins were crystallized, and anti-galactan TEPC 601 Fab, and an anti-levan EPC 109 Fab. The TEPC 601 crystals have been shown to be isomorphous with another anti-galactan, J539. The EPC 109 crystals are marginally too small for an x-ray diffraction investigation at present, and we are trying to make them bigger.

2. Considerable effort has been put into an attempt to determine the crystal structure of J539 Fab. Two heavy atom derivatives have been examined and the heavy atoms located. However, these are not sufficient for a structure determination and a search is continuing for more derivatives. Search techniques, using the known structure of McPC 603 have given some encouraging results, but have not yet led to a complete solution of the structure.

3. The sequence variations in a number of mouse myeloma proteins that bind phosphorylcholine have been examined in the light of the 603 three-dimensional structure. The key residues involved in binding phosphorylcholine in 603 are conserved in the other proteins, strongly suggesting that they all have the same binding site.

4. A model has been constructed of BS-5, an anti-type III polysaccharide antibody from a rabbit. The model building assumed an invariant framework part of the molecule on which the hypervariable loops were constructed based on the known sequence. The model exhibited a large cleft in the complementarity region with dimensions such that it could bind a hexasaccharide molecule. Attempts to crystallize intact rabbit antibodies (in collaboration with Dr. J.C. Jaton of Basel) have been partly successful in the very tiny crystals of one of the anti-type III polysaccharide antibodies have been obtained.

5. Data have been collected for another heavy atom derivative of McPC 603 in an attempt to improve the map.

Proposed Course: 1) The crystal structure of McPC 603 will be refined at as high a resolution as possible.

2) The investigation of J539 will be continued.

3) Further attempts will be made to obtain crystals of other mouse myeloma proteins and rabbit antibodies.

Significance to Bio-medical Research and the Program of the Institute:

An understanding of the molecular basis of interaction between antibody and antigen is fundamental to our understanding of the immune response.

Publications:

Richardson, J.S., Richardson, D.C., Thomas, K.A., Silverton, E.W. and Davies, D.R.: Similarity of Three-Dimensional Structure Between the Immunoglobulin Domain and the Copper, Zinc Superoxide Dismutase Subunit. J. Mol. Biol. 102, 221-235, 1976.

Davies, D.R. and Padlan, E.A.: Correlations Between Antigen-Binding Specificity and the Three-Dimensional Structure of the Antibody Combining Sites. In Krause, R. and Haber, E. (Eds.): The Future of Antibodies in Medicine. New York, Raven Press, 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 35000-12 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Chemical and Structural Investigations of Nucleic Acids and Related Substances

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

PI:	H. Todd Miles	Chief, Sec on Org. Chem.	LMB	NIAMDD
OTHER:	Frank B. Howard	Research Scientist	LMB	NIAMDD
	Girjesh Govil	Visiting Scientist	LCP	NIAMDD
	Yasuhiro Honda	Visiting Fellow	LMB	NIAMDD

COOPERATING UNITS (if any)

E. D. Becker, Laboratory of Chemical Physics, NIAMDD

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Organic Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3.5	1.5

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

This project has the long range objective of studying the chemistry and structure of nucleotides, polynucleotides and nucleic acids and the application of these studies to the understanding of biochemical and biological processes. Topics of current interest include: (1) structure and reactivity of polynucleotides and the relation between the two; (2) stabilization of ordered forms of nucleic acids and polynucleotides; (3) spectroscopic studies of polynucleotides: infrared, ultraviolet, and circular dichroism; (4) design and synthesis of new polynucleotides having properties required for the foregoing studies.

PHS-6040
(12-75)

Project Description:

Poly(G) and Poly(G)·Poly(C). Though G·C interactions are central to studies of nucleic acids, it has generally not been possible to use the interaction of poly(G) and poly(C) in a simple way as a model for this pairing. The principal impediment to studies of the interaction has been the high stability of the poly(G) self-structure (Tm >100).

The studies described below help to provide better understanding and control of the forms in which poly(G) occurs and of its interaction with poly(C).

Multiple Ordered Forms of Poly(G). We have found that there are two (or more) ordered forms of poly(G) which are quite distinct in the infrared spectra and, to a lesser extent, in their circular dichroism. We have designated the first form I, obtained by freezing and thawing a solution of the polymer. This has a strong carbonyl band at high frequency (in the range 1693-1690 cm^{-1} depending on method of preparation) and weak ring vibrations at 1610 and 1585 cm^{-1}. This material is progressively converted on standing to form II, having a carbonyl maximum at much lower frequency (1674 to 1667 cm^{-1}, depending on conditions) and ring vibrations at 1591 and ∿1578 (shoulder). The conversion is slow for undialysed polymer, having a half time of roughly 10 hours and requiring a week or more for completion. Conversion of dialysed polymer has $t_{1/2} \approx 1$ hour and is largely complete in a day. Mg^{++} appears to increase the conversion rate of the dialysed polymer. The I → II conversion is irreversible except by freezing and thawing the solution. Heating form I accelerates conversion to form II. Heating form II in the range 95°-100° causes little change in the infrared spectrum. The material is less than 5% melted under these conditions (e.g. 0.02 M poly(G), Na^+, no added counterion). Three different preparations of poly(G) having average chain lengths (end group determinations) of 44, 180, and 220, respectively all had essentially the same properties. The pH of the solution over the range 4.5-8.5 had no major effect on formation of I or its conversion to II though the rate of conversion appeared to be somewhat faster at pH 4.5 than at 8.5.

Electrostatic destabilization of poly(G). Formation of single stranded poly(G) at high temperature. We have previously found that $^+NEt_4$ counterion selectively destabilizes triple helices in comparison with double helices (JBC 246, 7073 (1971)), and attributed the result to the higher charge density of the former and to less effective counterion screening by $^+NEt_4$ than by Na^+. Since poly(G) is very probably four-stranded (Zimmerman et al., 1975; Arnott et al., 1975), its charge density is higher than that of triple helices and should be susceptible to destabilization by the same method. Poly(G) was converted to the $^+NEt_4$ salt by ion exchange chromatography and its infrared spectra observed as a function of temperature. A broad but cooperative melting was observed over the range ∿40°-100°, with Tm = 65°. At 98° the polymer appeared to be over 90% in the single stranded form, as judged by the characteristic

ring vibrations at 1576 and ∿1568 (shoulder). The melting is reversible, and the spectrum of form II reappears on cooling. This is the first time poly(G) of reasonably high MW has been obtained in single-stranded form.

Though circular dichroism of forms I and II is similar, there is a significant difference in that form I has a broad but resolved peak with λ_{max} 280 nm, which appears only as an unresolved shoulder of lower intensity between ∿270 and 300 nm in form II. The CD spectra of both forms are quite different from that predicted theoretically for poly(G) in a recent paper by Cech and Tinoco (Nucleic Acids Research 3, 399 (1976)). On the basis of measured and calculated poly(I) CD spectra and a predicted poly(G) spectrum, these authors concluded that the structure of poly(G) proposed by Arnott et al. was correct and that of Zimmerman et al. was not. Since the actual poly(G) spectra are quite different in their relevant features from the predicted one, we feel that the calculations do not provide a sound basis for reaching the structural conclusion for which they were employed with respect to poly(G).

Poly(G)·Poly(C). Commercial preparations of poly(G) (three suppliers) do not react to a major extent with poly(C) at 25° even after standing for days or weeks. After extensive dialysis, however, the reaction proceeds with a half time for G·C formation of 1 to 2 hours, but requires 1 or more days to reach a point at or near completion.[1] The reaction often appears to stop at a point about 5% to 10% away from completion as judged by the characteristic (and resolved) G·C ring vibration at 1591 cm^{-1}. Once formed, the poly(G)·poly(C) helix cannot be melted in D_2O in the presence of Na^+ (0.05 M), and the infrared spectrum shows no significant changes between 30° and 100°.

Poly(G)·poly(C) can be reproducibly prepared, even from the unreactive, non-dialysed polmers, by heating the homopolymers to 100° for a few minutes and allowing them to cool to 25°. The helix prepared in this way (and in the other ways described here) is well characterized by the frequency, intensity, and band shape of 7 infrared bands between 1750 and 1490 cm^{-1}.

We have examined the properties of poly(G)·poly(C) in mixtures of D_2O and dimethylsulfoxide (DMSO) both to determine the suitability of such mixed solvents for infrared spectroscopy and to determine whether the thermal stability of r(G)·r(C) can be conveniently lowered with organic solvents. Spectra observed with 50%, 60%, 70% and 80% DMSO (v/v) were essentially the same as that of r(G)·r(C) in pure D_2O, except that the C ring vibration at 1625 cm^{-1} is less well resolved from the carbonyl band at 1647 cm^{-1} in higher concentrations of DMSO.

---

[1] We have made consistent observations of this rather slow rate by three different spectroscopic methods on several different preparations of poly(G). We consider a contrary report (Pochon and Michelson, PNAS, 53, 1425 (1965)) of rapid and complete reaction to be in error.

Poly(G)·poly(C) in mixtures of DMSO and D_2O undergoes sharp, cooperative thermal transitions (breadth ∿10°) in a convenient temperature range: Tm 88° (50% DMSO); 72° (60% DMSO); 52° (70% DMSO). Examination of the spectra, however, shows that these are not simple helix → coil transitions. The poly(C) is converted to its random coil form, but the poly(G) is converted to an ordered form, which undergoes no further change between the temperature of the original transition and 100°. This form of poly(G) differs from the monomer GMP or single-stranded poly(G)(see previous section) in having ring vibrations at 1588 and 1571 cm^{-1} rather than 1580 and ∿1568 cm^{-1}. A more striking difference is the low absorbance which is only about a third as intense as that of the monomer or the single stranded form. The transition is irreversible, and r(G)·r(C) is not reformed on cooling the solution. If the ordered form of poly(G) described here is four-stranded, the observed thermal transitions can be formulated as follows:

$$4\ \text{poly(G)}\cdot\text{poly(C)} \rightarrow 4\ \text{poly(C)} + [\text{poly(G)}]_4$$

A similar thermal transition has been reported for poly($8NH_2G$)·poly(C) at pH 10 (Hattori et al., Biochemistry 14, 5033 (1975)), but at that pH the G·C helix underwent a second thermal transition to form the enolate anion of the single-stranded polymer.

Though several of the methods described above can produce poly(G)·poly(C) from the homopolymers and show that interaction is complete, the reaction is not reversible under any of these conditions. In pure aqueous solution dissociation of G·C does not occur below the boiling point of the solvent. In a destabilizing organic solvent (DMSO), dissociation of G·C does occur in an accessible temperature range, but the product is not single-stranded poly(G), and the reaction is largely irreversible on cooling. By replacing Na^+ counterion with Et_4N^+, however, these last properties of r(G)·r(C) are changed to resemble more closely those of other polynucleotide helices. In 0.1 M Et_4N^+, for example, r(G)·r(C) melts cooperatively with production of single-stranded poly(G) and poly(C):

$$\text{poly(G)}\cdot\text{poly(C)} \rightarrow \text{poly(G)} + \text{poly(C)}$$

The Tm is 82° under this condition, and on cooling poly(G)·poly(C) is largely reformed. There is often about 10% of G·G present after cooling, but in some runs, reformation of G·C was essentially 100%. We have found use of the Et_4N^+ salt of pyrophosphate to be important in achieving both complete dissociation and reformation of G·C, possibly because it protects against accidental metal ion contamination. (Howard, Frazier, and Miles).

Structure of poly(8BrA), an All-syn Polymer. We had previously reported the first synthesis of an all-syn polymer, poly(8BrA), and found that it has a highly ordered double helical structure, in contrast to published predictions that such a polymer would neither form double helices nor

undergo single strand stacking. The study is continuing with a combination of experimental and theoretical methods in collaboration with Dr. Girjesh Govil (LCP/NIAMDD). Potential energy calculations by Dr. Govil suggest that only one of three possible hydrogen bonding schemes is likely to lead to reasonable geometry of the ribose-phosphate backbone. A new synthesis of poly(8BrA) has been carried out, producing a polymer of somewhat lower Tm and much shorter chain length. We anticipate that this material will have advantages over the previous preparation for NMR investigation. Preliminary NMR spectra of this preparation are quite good. It will probably be possible to make from these a complete analysis of the ^{2}H spin-spin coupling constants and to obtain from these values information on the dihedral angles of the ribose-phosphate chain (Govil, Howard, Fisk, and Miles).

Publications:

Howard, F. B.; Frazier, J.; and Miles, H. T.: Poly(8-Bromoadenylic Acid): Synthesis and Characterization of an All-syn Polynucleotide. The Journal of Biological Chemistry, 250, 3951-3959, 1975.

Pinnavaia, T. J.; Miles, H. T.; and Becker, E. D.: Self-Assembled 5'-Guanosine Monophosphate. Nuclear Magnetic Resonance Evidence for a Regular, Ordered Structure and Slow Chemical Exchange. Journal of the American Chemical Society, 97, 7198, 1975.

Hattori, M.; Frazier, J.; and Miles, H. T.: Poly(8-aminoguanylic acid): Formation of Ordered Self-Structures and Interaction with Poly(cytidylic acid). Biochemistry, 14, 5033-5045, 1975.

Hattori, M.; Frazier, J.; and Miles, H. T.: Ordered Forms of 5'-8-Aminoguanylic Acid. Biopolymers, 14, 2095-2106, 1975.

Hattori, M.; Frazier, J.; and Miles, H. T.: The Structure of Triple-Stranded G·2C Polynucleotide Helices. Biopolymers, 15, 523-531, 1976.

Howard, F. B.; Frazier, J.; and Miles, H. T.: Poly-2-aminoadenylic Acid: Interaction with Polyuricylic Acid. Biochemistry, in press.

Miles, H. T.: "Infrared Properties of Polynucleotides." Fasman, G. D. (Ed.): Handbook of Biochemistry (ed. 3). Cleveland, CRC Press, pp. 604-623, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 35050-05 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Replication, Recombination and Repair of Microbial DNA

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

PI:	Jun-ichi Tomizawa	Chief, Sec on Mol Genetics	LMB	NIAMDD
OTHER:	Robert Bird	Staff Fellow	LMB	NIAMDD
	Tateo Itoh	Visiting Associate	LMB	NIAMDD
	Haruo Ohmori	Visiting Fellow	LMB	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Genetics Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	0

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

Mechanisms of replication, recombination and repair of DNA will continue to be investigated. In vitro studies on replication of plasmid DNA will be the major subject of investigation. Mechanisms of initiation, propagation and termination of replication of circular plasmid DNAs will be studied with the cell free system we have developed.

PHS-6040
(12-75)

Project Description:

Replication of closed-circular colicin E1 plasmid (Col E1) DNA can be initiated and completed in extracts of Escherichia coli. The major products of in vitro replication are completely replicated molecules and a unique type of early replicative intermediate containing a newly synthesised DNA fragment(s) in a small replication loop. The fragment has an average length of approximately one fifteenth of the unit length of the plasmid molecule and has a sedimentation constant of approximately 6 S. The replicated region of the intermediate consists of either one double-stranded branch and one single-stranded branch or two double-stranded branches. These intermediates accumulate in a reaction mixture containing 10% glycerol. Synthesis of the intermediates is inhibited by rifampicin but most of the intermediates can complete replication in the presence of rifampicin. We have studied the synthesis and fate of 6S DNA fragments formed on the parental heavy (H) strands and those formed on the parental light (L) strands of early replicative intermediates. The results show that the first synthesis of a DNA fragment is initiated at a specific site on the H strand and depends on the function of E. coli DNA-dependent RNA polymerase. Subsequent synthesis of the DNA fragment on the L strand does not involve the RNA polymerase.

A direct proof of the attachment of RNA to 6S L-DNA was shown by the transfer of ^{32}P-label in the DNA derived from [α-^{32}P]dNTPs to one of 2'(3') NMP after alkaline hydrolysis of 6S L-DNA. The base sequence at the RNA-DNA junction is specific but the specificity is not absolute. More than one third of the transfers are dT to rG and no significant transfer to rA was observed. The results indicate the presence of a preferred transition point as well as ambiguity at a single transition point. Preincubation of Col E1 DNA with purified bacterial RNA polymerase allowed some synthesis of Col E1 DNA in the presence of rifampicin. Some RNA synthesized by RNA polymerase was found to be attached to 6S L-DNA, indicating that RNA synthesized by bacterial RNA polymerase contains the primer for synthesis of the DNA.

In vitro Col E1 DNA replication can be carried out by bacterial functions without participation of any plasmid functions. Studies on necessary bacterial functions have been continued. DnaC(D) gene function was shown to be necessary for synthesis of 6S H-DNA but not of 6S L-DNA.

A cleavage map of Col E1 DNA by restriction enzymes was constructed. The region involving the origin of replication was finely mapped and the fragment which include the origin of replication was isolated. The fragment has approximately 40 base pairs. We are ready to sequence them.

With the in vitro system, the mechanisms of segregation of daughter molecules upon completion of replication of circular molecules can be studied. In colaboration with Dr. Y. Sakakibara (NIH, Japan) we studied mechanism of formation of catenanes which consist of two interlocked

circular molecules. It is concluded that two daughter molecules or a catenane are formed as the alternative products upon completion of replication of a circular molecule.

Publications:

Tomizawa, Jun-ichi: Two distinct mechanisms of synthesis of DNA fragments on colicin E1 plasmid DNA. Nature, 257, 253-254 (1975).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 36002-02 LMB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Novel Recombination Systems

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

PI: J. L. Rosner Research Biologist NIAMDD, LMB

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Microbial Genetics Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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

A gene for chloramphenicol-resistance (cam), derived from an R-factor, is present on a sequence of DNA which is translocatable to diverse replicons (e.g. coliphages P1 and λ and the Escherichia coli chromosome itself) in the absence of known recombinational functions or sequence homology. The basis of this extraordinary recombinational activity is being studied by genetic analysis and electron microscopy of heteroduplex DNA.

A second feature of this "translocon" is that it gives rise to deletions at high frequency resulting in the loss of the cam gene from the sequence. Some of the deletions are wholly within the translocon while others extend into the phage DNA. Particular attention is being given to a phage which does not lose the cam gene at high frequency. This phage is deleted for a portion of the phage chromosome adjacent to the translocon. The extent to which the deletion extends into the translocon is being studied.

PHS-6040
(12-75)

Project Description:

The purpose of this project is to understand in molecular terms the extraordinary ability of certain genetic elements ("translocons") to recombine with apparently unrelated DNA sequences in the absence of known recombination systems. The cam translocon is a sequence of about 2600 base pairs, a portion of which (about 600 b.p.) is a gene determining resistance to chloramphenicol (cam gene). Originally present on an R-factor, this translocon has recombined with bacteriophages P1 and λ and with the Escherichia coli chromosome itself. In the case of λ, the translocon will insert itself into at least two distinct sites on λ. About 1% of the time that translocon has integrated into the E. coli chromosome, the insertion has led to a recognizable mutation presumably due to insertion within a gene. Since this process of translocation does not require any known bacteriophage or bacterial recombinational activities, it is thought that the translocon itself possesses a mechanism for self-insertion.

Another unusual aspect of the cam translocon is that portions of it are deleted at high frequency. Two processes appear to operate the first, dependent upon known recombinational functions, appears to involve recombination between homologous sequences present at each end of the translocon and bracketing the cam gene. The second, independent of known recombinational functions, appears to be the generation of deletions by a portion of the translocon. In some cases, the deletion eliminates the cam gene and an adjacent portion of the bacteriophage chromosome. In other cases, the cam gene remains intact but an adjacent segment of the bacteriophage DNA is lost. These variants are now being studied by electron microscopy to correlate translocatability, drug resistance and deletion-formation with the different portions of the translocon.

Publications:

Rosner, J. L.: Specialized transduction of pro genes by coliphage P1: Structure of a partly diploid P1-pro prophage. Virology, 67: 42-55, 1975.

Gottesman, M. M. and Rosner, J. L.: Acquisition of a determinant for chloramphenicol resistance by coliphage lambda. Proc. Nat. Acad. Sci. USA 72: 5041-5045, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 36051-08 LMB

PERIOD COVERED
July 1 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Genetics and structure of the oncogenic virus, SV40

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

PI:	Robert G. Martin	Chief, Sec on Microbial Genetics	LMB NIAMDD
OTHER:	Jeffrey Anderson	Research Associate	LMB NIAMDD
	Maria DiLauro	Visiting Fellow	LMB NIAMDD
	William Brockman	Research Associate	LMB NIAMDD
	Stefanie Stein	COSTEP	LMB NIAMDD
	Carol Edwards	Staff Fellow	LMB NIAMDD

COOPERATING UNITS (If any) David Livingston, Asst. Prof., Harvard Medical School

Vincent Bono	Head, Mol. Biol. & Methods Dev. Sec.	LMCB NCI
Peter T. Mora	Head, Macromolecular Biol. Sec.	LCBGY NCI
George Khoury	Head, Virus Tumor Biol. Sec.	TDTV NCI
Chungming Chang	Visiting Fellow	LCBGY NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Microbial Genetics Section

INSTITUTE AND LOCATION
NIAMDD, LMB, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4-1/3	4-1/3	0

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

It is the long range goal of this project to discover how SV40 causes transformation. SV40 is a small DNA virus of the papova virus group. Papovaviruses have been implicated in cancer and progressive multifocal leukoencephalopathy. Current topics of interest are (1) the cell cycle in normal and SV40 transformed cells, (2) the origin and structure of the tumor specific transplantation antigen, (3) the structure of SV40 chromatin, (4) regulation of the synthesis of SV40 tumor antigen, (5) the structure and function of the tumor antigen, and (6) the mode of DNA replication in normal and transformed cells.

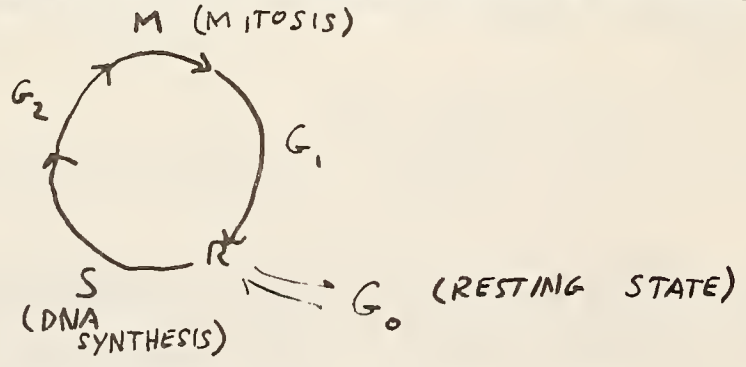
Project Description:

Objective: To analyze the molecular basis of transformation by SV40 using conditional lethal mutants.

Major Findings: (a) Further data has been obtained to demonstrate that the A gene of SV40 is required for the maintenance of transformation (Brockman and Martin), and that the T antigen is the gene product of the A gene. Additional transformants by tsA mutants have been obtained and analyzed. In addition revertants of these ts mutants have been obtained from Drs. Thomas Shenk and Paul Berg and similarly examined. As expected the ts mutants, but not the revertants, are ts for transformation and the purified T antigen (TA) is temperature sensitive by DNA binding and complement fixation (Anderson and Martin in collaboration with D. Livingston).

(b) Very high levels of tumor specific transplantation antigen (TSTA) activity have been demonstrated in nuclei of transformed cells. It had been thought that TSTA was only associated with plasma membranes. In collaboration with D. Livingston we have shown that TSTA from nuclei co-purifies with the TA and have proposed that the two are identical. Since the TSTA associated with plasma membranes does not contain TA activity we assume that TA is a precursor of this activity (Anderson and Martin associated with C. Chang, P. Mora and D. Livingston).

(c) Cells which are transformed by tsA mutants can enter a resting state, G_o, at the nonpermissive temperature but not at the permissive temperature. An analysis of the length of time to initiate DNA synthesis after amino acid or serum depletion of cells has led us to propose that normal cells leave the cell cycle under these conditions according to the scheme:



If correct this model supports our previous suggestion that SV40 transformation is the result of the TA acting at the initiation of DNA synthesis. The level of TA at the point R (restriction point) would then be crucial in driving an SV40 transformed cell into S phase when a normal cell would enter G_o as a result of serum or amino acid deprivation (Martin and Stein).

(d) Partially purified TA is known to react site specifically with SV40 DNA. We have purified SV40 chromatin from infected monkey cells and have shown (1) that TA binds (although less efficiently than DNA) to this

chromatin and (2) that the histone complement of this chromatin assumed to be packed in "ν" bodies, is randomly distributed across the SV40 chromatin (DiLauro and Martin).

(e) DNA radioautography of normal and SV40 transformed cells has been performed. Preliminary results suggest that transformed cells have more points of DNA initiation (Martin).

(f) Late ts mutants of the D group were postulated to be defective in "uncoating". We have now demonstrated that these mutants fail to make early RNA (Avila, Saral, Martin and Khoury).

The significance of this research to biomedical science is that it may lead to a fundamental understanding of some parameters of normal cell growth and how this growth is preturbed on transformation by SV40.

Work in progress includes an attempt to demonstrate that TA is cell cycle dependent in intermediate transformants (in collaboration with V. Bono) and to purify large quantities of pure TA so that its chemical properties can be studied.

Publications:

Martin, R. G. and Anderson, J. L.: Death and Transformation. In Yuhas, J.M., Tennant, R. W., and Regan, J. A. (Ed.): Biology of Radiation Carcinogenesis. New York, Raven Press, 1976, pp. 287-300.

Anderson, J. L. and Martin, R. G.: SV40 transformation of mouse brain cells: Critical role of gene A in maintenance of the transformed phenotype. J. Cell Physiol. 88: 65-76, 1976.

Martin, R.G. and Stein, S.: The resting state in normal and SV40 transformed Chinese hamster lung cells. Proc. Nat. Acad. Sci. USA, in press, 1976.

Avila, J., Saral, R., Martin, R. G., and Khoury, G.: The temperature-sensitive defect in SV40 group D mutants. Virology, in press, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 36101-03 LMB

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Energy conversion in biology, especially muscle contraction

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

PI:	Terrell L. Hill	Chief, Section on Theoretical Mol. Biol. LMB	
OTHER:	Igor Plesner	Visiting Scientist	LMB NIAMDD
	Yi-der Chen	Visiting Associate	LMB NIAMDD

COOPERATING UNITS (if any)

Evan Eisenberg H LB

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Theoretical Molecular Biology Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2-1/4	2-1/4	0

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

In previous work, we have related the mechanics of muscle contraction to the biochemical kinetics, the latter being expressed in terms of "given" rate constants. We have begun, during the current year, an attempt to go one step more deeply into the kinetics by studying the molecular theory of the rate constants themselves. One part of this work deserves special comment: it extends the range of validity of Eyring's widely used rate theory (for gas reactions) to incude solution reactions and diffusion effects.

We have also investigated the connection between free energy levels of a cycling macromolecule (e.g., an enzyme) at steady-state and the kinetics of the cycle. The entire subject of steady-state kinetics and thermodynamics, and its relation to free energy transduction in biology, is being treated in a monograph that will be completed during the current year.

PHS-6040
(12-75)

Project Description:

In previous work, we have related the mechanics of muscle contraction to the biochemical kinetics, the latter being expressed in terms of "given" rate constants. We have begun, during the current year, an attempt to go one step more deeply into the kinetics by studying the molecular theory of the rate constants themselves. The first three papers below are on this topic. The second of these papers deserves special comment: it extends the range of validity of Eyring's widely used rate theory (for gas reactions) to include solution reactions and diffusion effects.

The fourth and fifth papers below investigate the connection between free energy levels of a cycling macromolecule (e.g., an enzyme) at steady-state and the kinetics of the cycle. The entire subject of steady-state kinetics and thermodynamics, and its relation to free energy transduction in biology, is being treated in a monograph that will be completed during the current year.

Publications:

Hill, T. L.: Effect of rotation on the diffusion-controlled rate of ligand-protein association. Proc. Nat. Acad. Sci. USA, 72: 4918-4922, 1975.

Hill, T. L.: Diffusion frequency factors in some simple examples of transition-state rate theory. Proc. Nat. Acad. Sci. USA, 73: 679-683, 1976.

Hill, T. L. and Eisenberg, E.: Reaction free energy surfaces in myosin - Actin - ATP systems. Biochemistry, 15: 1629, 1976.

Hill, T. L. and Simmons, R.M.: Free energy levels and entropy production associated with biochemical kinetic diagrams. Proc. Nat. Acad. Sci. USA 73: 95-99, 1976.

Hill, T. L. and Simmons, R.M.: Free energy levels and entropy production in muscle contraction and in related solution systems. Proc. Nat. Acad. Sci. USA, 73: 336-340, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 36102-05 LMB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Statistical thermodynamics of polynucleotide - complementary monomer interactions

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

PI: Terrell L. Hill Chief, Sec on Theoretical Mol Biol LMB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Theoretical Molecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

This project has been inactive during the current year.

5881

PHS-6040
(12-75)

Project Description:

The objective here is: (a) to clarify the thermodynamic procedures which should be used in comparing optical, calorimetric, and binding measurements on these systems; and (b) to develop and use, as a prerequisite, a theory of aggregation of monomers in solution.

Extensive work has already been done under both (a) and (b). Applications in the future to particular systems await the completion of joint experimental work by Dr. Philip Ross and Dr. Thomas Schleich (Univ. of Calif., Santa Cruz).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 36103-05 LMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Ion transport across the nerve axon membrane

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

PI:	Terrell L. Hill	Chief, Sec on Theoretical Mol Biol	LMB	NIAMDD
OTHER:	Yi-der Chen	Visiting Associate	LMB	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Theoretical Molecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	3/4	0

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

In our previous work, we were concerned with the question of how to calculate the concentration noise power spectrum of an ensemble of multi-state linear kinetic systems when the rate constants of the systems are assumed to be known. We have used a standard eigenvalue-eigenfunction method to solve the differential equations which govern the regression of the means and derived the noise power spectrum as a function of the eigenvalues and eigenfunctions of the relaxation matrix of the system. In our current work, we have obtained an equation which relates the noise spectrum matrix of the fluctuations directly to the relaxation matrix of the means. As a result, the noise power spectrum can be calculated through matrix operations without the necessity of an eigenvalue-eigenfunction calculation. The present formalism is particularly useful in the evaluation of kinetic rate constants when the noise spectrum data of concentration fluctuations are given. Possible applications to biochemical systems have been considered.

PHS-6040

Project Description:

Our general objective in this work is 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 conformation changes.

During the current period, we have pursued the noise problem somewhat further. Since noise involves the decay of spontaneous fluctuations, the kinetic parameters obtained from noise measurements are the same as those obtained from ordinary kinetic studies or chemical relaxation measurements. But, due to the fact that they can be carried out at steady-state or at equilibrium without applying any perturbation to the system as a relaxation method usually does, noise measurements may prove to be very useful in in vivo studies of biological systems.

In our previous work, we were concerned with the question of how to calculate the concentration noise power spectra of an ensemble of multi-state linear kinetic systems when the rate constants of the systems are assumed to be known. We have used a standard eigenvalue-eigenfunction method to solve the differential equations which govern the regression of the means and derived the noise power spectrum as a function of the eigenvalues and eigenfunctions of the relaxation matrix of the system. In our current work, we have obtained an equation which relates the noise spectrum matrix of the fluctuations directly to the relaxation matrix of the means. As a result, the noise power spectrum can be calculated through matrix operations without the necessity of an eigenvalue-eigenfunction calculation. The present formalism is particularly useful in the evaluation of kinetic rate constants when the noise spectrum data of concentration fluctuations are given. Possible applications to biochemical systems have been considered.

Publications:

Chen, Yi-der: A matrix method for fluctuations and noise in kinetic systems. Proc. Nat. Acad. Sci. USA, 72: 3807, 1975.

Chen, Yi-der: Differentiation of channel models by noise analysis. Biophy. J. (in press) 1976.

Annual Report of the

Mathematical Research Branch

National Institute of Arthritis, Metabolism, and Digestive Diseases

Improvement of the efficiency of nearest neighbor algorithms is being investigated. Finding the nearest neighbor in an n dimensional space is theoretically straightforward; however, if the dimension is high, the computing time and expense of the brute force method becomes prohibitive. One anti-tumor drug structural description, for example, used over 400 dimensions, and about 300 known drugs. The efficiency of known algorithms drops rapidly with increasing dimension. Two methods of improving efficiency are being studied. First, to approximate the n dimensional space by an r dimensional space, $r < n$, and find a better lower bound for the distance between points. Second, to rotate coordinate axes so that for a given value of r, the best lower bound can be achieved. Simulations of searches through a collection of twenty points in a ten dimensional space have been conducted and results are promising. Further studies are being conducted to provide an analytical measure of the improvement achievable by these new algorithms. (Ms. R. B. Marimont, Associate member, MRB).

During the past year, in an extension of earlier work on kidney models, a multinephron multisolute model of the whole kidney has been developed. This model takes account of the cortical and medullary distribution of nephrons and capillaries and includes salt, water, and urea transport, and both hydrostatic and oncotic pressure. With this model it should be possible to analyze several aspects of renal function such as the effect of urea cycling on passive salt transport out of thin ascending limb of Henle, and the effects of changes in the distribution of blood flow between cortex and medulla.

In all of these computer simulations the need for a steady effort to improve numerical methods has been apparent. Toward this end we have continued our cooperative and collaborative programs with mathematicians at the University of Maryland, Louisiana Polytechnic Institute, and SUNY, Stony Brook, N. Y. We also have spent considerable time comparing the stability and efficiency of various algorithms. In order to promote an exchange of information on these problems we organized two sessions on numerical methods in kidney modeling as part of the 1976 Summer Computer Simulation Conference, which will take place in Washington, D. C.

A review of the past, present, and future of kidney modeling was carried out as part of the NIAMDD survey of research needs in nephrology. From this survey it seemed clear that models of the mammalian kidney have reached a level of sophistication such that realistic simulation of several aspects of renal function is now possible and simulation of additional features should be possible in the near future. These models should provide the basis for the estimation of transmembrane transport parameters from data on whole kidney function. If this goal can be reached, it will give new interpretations of both normal and abnormal renal function. (Dr. J. L. Stephenson and Mr. R. Mejia, NHLI, Associate members, MRB).

A mathematical model describing the facilitated diffusion of oxygen by hemoglobin was developed and a numerical analysis was implemented for a large scale digital computer. This has been reported in a previous year. The research is extended into a qualitative analysis of the system of differential equations involved. The analysis is done in terms of: 1. two diffusion competing channels, namely the free and the combined oxygen forms; 2. the chemical kinetics involved in the passage of one form into the other and 3. the influence of the boundaries, impermeable to the combined form. A result of this analysis shows that the limitation to the facilitation is essentially due to the unloading of oxygen (transfer of oxygen from the combined to the free form) near the low oxygen boundary. This aspect was incorporated into a simplified description of facilitation that resulted in an algebraic equation formulation. This formulation contains explicitly the parameters of the system. Its numerical solution is easily obtainable with an electronic desk calculator, bypassing the need of a large scale digital computer. A novel feature is that the extent of the replacement of a nonlinear chemical kinetics by a linear one is part of the solution to the problem itself and cannot be decided upon, a priori. (Dr. J. Gonzalez-Fernandez and Mrs. S. Atta).

As reported last year, for a simplified FitzHugh-Nagumo nerve conduction equation there exist multiple traveling wave solutions. This leads to questions of stability for such solutions. A new and appropriate notion of stability, viz. spatial stability, has been formulated and an explicit analysis presented (Biophys. J. 15, 975, 1975) along with the formal extension to a class of conduction equations (J. Math. Biol. 2, 205, 1975).(Dr. J.Rinzel)

Under constant current stimulation, a neuron may propagate a repetitive train of action potentials along its axon. For such repetitive firing, the current strength I must typically be in some appropriate range $I_1 < I < I_2$. Outside this range, time independent steady state behavior is usually achieved. Features of this qualitative picture are reflected by results for the corresponding mathematical problem. The following results are obtained for the FitzHugh-Nagumo nerve conduction equation and are expected to extend to a class of models. For each value of I there is a spatially nonuniform steady state solution. For small or large I it is a stable solution but for an intermediate range of I values it is unstable. This intermediate range thus lies interior to the range of I values for repetitive activity. Furthermore time periodic solutions bifurcate from the steady state at upper and lower critical values of I. These bifurcation phenomena provide insight for the experimental observation that the firing frequency jumps discontinuously as I passes through the values I_1 and I_2. (Dr. J. Rinzel).

Interesting nonlinear wave propagation phenomena have been observed experimentally in cardiac Purkinje fibers, which form the specialized electrical conduction system of the heart. These effects, such as conduction of one pulse through a damaged region for every two or three incident pulses or conduction through a damaged region in only one direction, are thought by the experimenters to be intimately connected with the nature of certain heart disorders. Earlier work (Doctoral thesis, Univ. California, Berkeley, Jan. 1976) proposed a mathematical model of this system and was able to reproduce

some of the experimentally observed effects by numerical simulation. Further work is being pursued to extend these results. (Dr. R. Miller).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 13,000-03 MRB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mathematics of kinetics and reaction-transport systems

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

PI: John Z. Hearon Chief, MRB MRB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	2.0	.6

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

This project continues to focus upon applications of linear algebra, matrix and vector space methods to problems in biology. Applications are heavily involved in compartmental analysis; linear control theory, and generalized kinetics. No small part of the project is devoted to meeting purely mathematical problems which arise on their proper abstract grounds.

PHS-6040
(12-75)

1. Bounds for roots of a polynomial.

The following is an outgrowth of the work reported last year on the Leslie population matrix. A theorem of the greatest theoretical import states that the modulus of every root of a matrix is bounded between the maximum and minimum singular values. As a practical matter it is not very simple to determine the singular values. It has been shown that for a matrix in companion form, the maximum and minimum singular values can be determined explicitly as the roots of a quadratic equation. Thus the required bounds are furnished by elementary algebra. Since the roots for every polynomial coincide with those of the corresponding companion matrix, the method applies to an arbitrary polynomial.

2. Comparison of vector norms.

It is well known that on a finite dimensional space all norms are equivalent although the numerical constants specifying this for a given pair of norms are not easy to come by. It has been shown that no standardized norm can exceed the Hölder h_1-norm, and a corresponding theorem for an arbitrary norm easily follows. The result is a boundary of an arbitrary norm between two Hölder norms in terms only of the norm of the unit basic vectors. Special choices of the norm yield theorems having nothing to do with norms. For example, if we choose an abritrary ellipsoidal norm we obtain bounds on an arbitrary positive quadratic form.

3. Characterization of ϕ-isometry for the Hölder norms.

The "length" or Euclidean norm of a vector has great intuitive appeal. In practice it is not especially convenient nor is the corresponding induced matrix norm. An isometry is a linear operator which leaves the Euclidean norm unchanged. For an arbitrary norm ϕ, we call an operator K a ϕ-isometry if $\phi(Kx) = \phi(x)$ for all x. The characterization of a ϕ-isometry for arbitrary ϕ is an important open problem in operator theory. We have produced a characterization for the case that ϕ is an arbitrary Hölder norm: Let k_i and k_j be arbitrary columns of K and e_i and e_j the corresponding unit coordinate vectors. Then K is a ϕ-isometry if and only if $\phi(\alpha k_i + \beta k_j) = \phi(\alpha e_i + \beta e_j)$ for arbitrary complex scalors α, β and every i, j.

Publications:

Hearon, J. Z.: Properties of the Leslie population matrix. Bull. Math. Biol. 38: 199-203, 1976.

Hearon, J. Z.: Nonsingular solutions of TA - BT = C. Lin. Algebra and Appl. (in press).

Hearon, J. Z.: Bounds on the roots of a polynomial obtained from the companion matrix. Math. Mag. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 13,001-03 MRB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mathematical formulations and analysis relevant to experimental neurophysiology.

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

PI:	Wilfrid Rall	Senior Research Physicist	MRB NIAMDD
OTHER:	John Rinzel	Research Mathematician	MRB NIAMDD
	Maurice Klee	Staff Fellow	MRB NIAMDD
	Robert Miller	Staff Fellow	MRB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.7	0.3

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

For several years, this project has been concerned with constructing, developing and testing a group of interrelated mathematical models, relevant to experimental neurophysiology and neuroanatomy. Together these models provide a theory that can account for various sequences of events in the soma and dendritic branches of a single neuron, and for field potentials generated by certain cortical populations of neurons. Computational experiments performed with these models provide theoretical predictions that have been compared with experimental results obtained by colleagues with motoneurons of cat spinal cord, and with the mitral cell and granule cell populations of rabbit olfactory bulb. Resulting interpretations contribute to understanding of dendritic synaptic input and of dendro-dendritic synaptic interactions.

Some of these results have appeared in the Biophysical Journal (9:1483-1508 and 1509-1541, 1969), (13:648-688, 1973), (14:731-757 and 759-790, 1974) and in the Journal of Neurophysiology (30:1072-1193, 1967) and (31:884-915, 1968). A comprehensive chapter, entitled "Core conductor theory and cable properties of neurons" will appear in The Nervous System, Vol. I, Cellular Biology of Neurons, edited by E. R. Kandel, to be published by The American Physiological Society in 1976.

1. (With Dr. Klee). Presentation and analysis of the characteristics of electric fields generated by activity in a single neuron and in open and closed cortical arrangements of neuron populations. The preliminary draft (prepared by Dr. Klee before his abrupt departure) required extensive revision to correct errors of fact and of emphasis. The resulting MS, submitted for publication, provides computed potential fields for several idealized cortical arrangements of neuronal populations and discusses their implications. These results provide confirmation and further understanding of the simple "potential divider model" introduced earlier by Rall and Shepherd.

2. (With Dr. Rinzel). Some discussion (but no final drafts) of two manuscripts intended to describe synaptic input to dendritic spines and possible implications for neuronal plasticity. Some of this has been presented in symposia and reviews; it is based upon our earlier theoretical results.

3. (With Dr. Miller). We are discussing modeling and computation relevant to synapses in tissue culture and in small systems of neurons.

4. Editorial interchange, acceptance and minor revisions of the chapter cited next.

Publications:

Rall, W.: Core conductor theory and cable properties of neurons. In Kandel, E. R. (Ed.): The Nervous System, Vol. I, Cellular Biology of Neurons. Washington, D.C., Am. Physiol. Soc., 1976 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 13,002-04 MRB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Mathematical description of the substrates transport in the capillary-tissue structures.

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

PI:	Jose M. Gonzalez-Fernandez	Research Mathematician	MRB NIAMDD
OTHER:	Susie E. Atta	Mathematician	MRB NIAMDD

COOPLRATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.4	3	.4

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

To develop mathematical models of the blood flow and transcapillary exchanges in capillary networks. An effort is being made to incorporate in the models the histological structure of capillary networks as well as different flow patterns from available experimental information. In this model the extraction of substrates with different chemical kinetics at the tissue site will be described. It is expected that this could be used in experimental situations where the extraction of different substrates are measured simultaneously, thus helping to infer the flow pattern features of the microcirculation. In particular a model of the diffusion-consumption of oxygen in striated muscle containing myoglobin (facilitated diffusion) is being developed and pertinent numerical results examined.

PHS-6040
(12-75)

A mathematical model describing the facilitated diffusion of oxygen by hemoglobin was developed and a numerical analysis was implemented for a large scale digital computer. This has been reported in a previous year. The research is extended into a qualitative analysis of the system of differential equations involved. The analysis is done in terms of: 1. two diffusion competing channels, namely the free and the combined oxygen forms 2. the chemical kinetics involved in the passage of one form into the other and 3. the influence of the boundaries, impermeable to the combined form.

A result of this analysis shows that the limitation to the facilitation is essentially due to the unloading of oxygen (transfer of oxygen from the combined to the free form) near the low oxygen boundary.

This aspect was incorporated into a simplified description of facilitation that resulted in an algebraic equation formulation. This formulation contains explicitly the parameters of the system. Its numerical solution is easily obtainable with an electronic desk calculator, bypassing the need of a large scale digital computer.

A novel feature is that the extent of the replacement of a nonlinear chemical kinetics by a linear one is part of the solution to the problem itself and cannot be decided upon, a priori.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 13,004-02 MRB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mathematical description of cellular neuroelectric signal transmission.

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

PI:	John Rinzel	Research Mathematician	MRB NIAMDD
OTHER:	Richard FitzHugh	Research Physicist	LB NINCDS
	Robert Miller	Staff Fellow	MRB NIAMDD

COOPERATING UNITS (if any)

Laboratory of Biophysics, NINCDS

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.3	.3

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

This project continues to focus on the formulation, analysis, and biophysical interpretation of mathematical models which describe various aspects of neuroelectric signaling for individual neurons. Among the topics of current interest are:

(i) integration of synaptic input delivered to the soma and dendritic branches of a neuron;
(ii) steady propagation of action potentials along axons;
(iii) threshold properties for repetitive firing of action potentials;
(iv) excitable membrane characteristics and firing patterns of bursting pacemaker neurons.

Mathematical models of these phenomena involve systems of nonlinear ordinary differential equations and parabolic partial differential equations. Solutions and their mathematical stability are determined by analytical and numerical methods. One aspect in the approach of this project is to expose the qualitative mathematical structure for classes of models by exploiting simple, yet reasonable, equations.

PHS-6040
(12-75)

1. Now complete are two articles [3, 5] which survey the analysis and biophysical implications of mathematical models for dendritic integration and axonal impulse conduction. The articles include results (obtained with Dr. Rall, MRB, in earlier papers) for transient and steady state response for input to a single branch location of a dendritic neuron model. The surveys also describe the traveling wave solutions for a simplified FitzHugh-Nagumo nerve conduction equation. Because there is a multiplicity of such solutions one is led to questions of stability for these solutions. In the context of signaling problems encountered in neurophysiology the appropriate notion of stability, spatial stability, of these solutions is formulated and an explicit analysis is discussed. These results and their formal extension to a class of nerve conduction equations are described in more detail in two recent papers [1, 2].

2. Under constant current stimulation, a neuron may propagate a repetitive train of action potentials along its axon. For such repetitive firing, the current strength I must typically be in some appropriate range $I_1 < I < I_2$. Outside this range, time independent steady state behavior is usually achieved. Features of this qualitative picture are reflected by results for the corresponding mathematical problem. The following results are obtained for the FitzHugh-Nagumo nerve conduction equation and are expected to extend to a class of models. For each value of I there is a spatially nonuniform steady state solution. For small or large I it is a stable solution but for an intermediate range of I values it is unstable. This intermediate range thus lies interior to the range of I values for repetitive activity. Furthermore time periodic solutions bifurcate from the steady state at upper and lower critical values of I. These bifurcation phenomena provide insight for the experimental observation that the firing frequency jumps discontinuously as I passes through the values I_1 and I_2.

3. (With Dr. Miller). Various types of neurons exhibit steady repetitive firing over a range of different frequencies. Correspondingly, nerve conduction equations typically have a one parameter family of periodic traveling wave solutions. The propagation speed θ of a periodic wave train solution depends upon its frequency ω. However the dispersion relation $\theta = \theta(\omega)$ is known for only one nerve conduction equation; a simplified FitzHugh-Nagumo equation which can be solved explicitly [3, 5]. Our goal is to study the dispersive aspect of nerve conduction for other models. For this we must first devise a stable and efficient numerical procedure to determine the wave train solutions and their dispersion relation for the other models.

4. (With Dr. FitzHugh). During repetitive activity, certain nerve cells fire in regular bursting patterns. A bursting pacemaker (e.g., Aplysia R15 cell) will spontaneously exhibit this behavior. Some investigators have proposed mathematical models for bursting behavior by modifying and expanding the four-variable Hodgkin-Huxley model for excitable membrane. Our approach is to formulate and study a simple qualitative model. For this we modify a basic two-variable FitzHugh-Nagumo equation and introduce a third variable. Preliminary numerical solutions obtained by analog and digital computation exhibit bursting cycles for certain values of the model parameters. Further

numerical simulations will study the onset and variation of bursting patterns with changes in model parameters.

Publications:

1. Rinzel, J.: Spatial stability of traveling wave solutions of a nerve conduction equation, Biophys. J. 15: 975-988, 1975.

2. Rinzel, J.: Neutrally stable traveling wave solutions of nerve conduction equations, J. Math. Biol. 2: 205-217, 1975.

3. Rinzel, J.: Simple model equations for active nerve conduction and passive neuronal integration, in Lectures on Mathematics in the Life Sciences, vol. 8 (S. A. Levin, ed.), Am. Math. Soc., Providence, R. I., 1976.

4. Rinzel, J.: Nerve signaling and spatial stability of wave trains, in Structural Stability, Catastrophe Theory and Their Applications in the Sciences. (P. J. Hilton, ed.) Springer-Verlag, New York. (in press).

5. Rinzel, J.: Integration and propagation of neuroelectric signals, in MAA Study in Mathematical Biology (S. A. Levin, ed.), Math. Assoc. America, Washington, D. C. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 13,011-01 MRB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mathematical models of electrical impulse propagation in excitable tissue

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

PI:	Robert Miller	Staff Fellow	MRB NIAMDD
OTHER:	John Rinzel	Research Mathematician	MRB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.4	.4	

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

We wish to gain insight into the nature of the propagation of electrical impulses in heart and nerve cells. In this project we use both analytical and computational techniques to investigate the behavior of several mathematical models of normal cells and cells that have been damaged by poison or disease. Work is currently proceeding in two general directions: 1) determination of dispersion curves for the FitzHugh-Nagumo equations and 2) numerical and analytical study of anomalous propagation of electrical impulses in damaged heart fibers.

1. (With John Rinzel). The FitzHugh-Nagumo model of active electrical behavior of nerve cells is known to possess periodic traveling wave solutions. These solutions correspond to periodic trains of pulses propagating along the cell. We wish to determine the manner in which the speed of such a train of pulses is related to its frequency. Such a relation is known as a dispersion relation. Standard methods for performing the calculations involved are thought to be inefficient, and new computational methods may have to be developed and tested.

2. Interesting nonlinear wave propagation phenomena have been observed experimentally in cardiac Purkinje fibers, which form the specialized electrical conduction system of the heart. These effects, such as conduction of one pulse through a damaged region for every two or three incident pulses or conduction through a damaged region in only one direction, are thought by the experimenters to be intimately connected with the nature of certain heart disorders. In my doctoral dissertation I proposed a mathematical model of this system and was able to reproduce some of the experimentally observed effects by numerical simulation. Through this model I hope to provide a conceptual framework for understanding this system. The work that has been done is now being written up for publication, and further work is being done to extend the earlier results.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 13,012-01 MRB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mathematical modeling of reaction kinetics for cellular arrays

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

PI:	John Rinzel	Research Mathematician	MRB NIAMDD
OTHER:	Barry Bunov	Staff Fellow	LAS DCRT

COOPERATING UNITS (if any)

Laboratory of Applied Studies, DCRT

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.8	

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

Mathematical models which allow for intracellular enzyme-substrate reactions and intercellular substrate diffusion are formulated. These models lead to systems of nonlinear ordinary differential equations which can exhibit multiple steady state and time periodic solutions. The goal of this project is to develop analytical and numerical methods to determine these solutions and their stability. Analytical characterizations for critical values of model parameters are sought through bifurcation theory.

PHS-6040
(12-75)

ANNUAL REPORT OF CLINICAL INVESTIGATIONS, NIAMDD

July 1, 1975 to June 30, 1976

Phillip Gorden, M.D., Clinical Director

The seven branches comprising Clinical Investigations have had no significant changes in personnel over the past year. Dr. Saul Rosen will be returning soon from a year's foreign assignment in England. The overall difficulty in recruiting Clinical Associates has not been reflected in NIAMDD. Our Associates have maintained a very high standard of patient care.

Considerable time has been spent by a number of our senior staff in planning the new Ambulatory Care Research Facility. This Institute has taken a major leadership role in implementing the goals set for the newly organized Medical Board of the Clinical Center. We have made space available on 9-West for the construction of an intensive care unit, a badly needed facility for the management of acute problems that result during the course of many studies in this Institute and others in the Clinical Center. This is a very significant part of an emerging feeling of the interdependence of all the Institutes in providing a high standard of medical care which is an absolute requirement for quality clinical research.

Annual Report of the Arthritis and Rheumatism Branch
National Institute of Arthritis, Metabolism and Digestive Diseases

Investigations in ARB cover the gamut of direct therapeutic studies on human patients, use of animal models to discover pathogenetic mechanisms and fundamental studies designed to develop new investigative tools and increase our knowledge about normal biochemical and immunologic mechanisms.

I. Therapeutic Trials

A. No satisfactory therapy exists for severe manifestations of Systemic Lupus Erythematosus (SLE) except for renal disease and even the latter does not respond to conventional therapy when the creatinine clearance is markedly reduced. A controlled trial of intravenous cyclophosphamide in patients with severe renal disease, and/or extra-renal manifestations of SLE has been initiated (J. L. Decker, P. H. Plotz, A. D. Steinberg) as part of a continuing search for effective therapy of this serious disease. Six patients have been admitted to the study so far but there are no significant findings to date.

In a separate study (J. L. Decker, P. H. Plotz, A. D. Steinberg, P. J. Rooney) of patients with diffuse glomerulonephritis and systemic lupus erythematosis alternative treatment programs involving corticosteroids, azathioprine and cyclophosphamide are being assessed. So far patients receiving no immunosuppressive drug have progressed to requiring renal dialysis or to death somewhat earlier than those receiving such drugs. However, toxicity in the absence of major long-term advantages over four years of follow-up suggest that such regimens should not be continued in the future.

B. Aspirin, a widely used drug in connective tissue disease, has been found to induce changes in renal function tests, thereby complicating assessment of renal function in patients on the drug. The basis of the aspirin effect is being studied (P. H. Plotz, R. Kimberly, J. R. Gill, Jr.).

II. Disease Mechanisms-Human

A. A variety of immunologic abnormalities have been observed in mice afflicted with a disorder closely resembling human systemic lupus erythematosus (see below). Several investigations on humans have been initiated to assess to what degree similar abnormalities occur with the human form of the disease.

American Indians showing an unusually high incidence of the disease (Sioux, Crow and Arapaho tribes) as well as others are being investigated (A. D. Steinberg, D. R. Budman, L. W. Klassen, J. P. Reeves, W. Glinski, C. Brady, E. B. Merchant). It has been found that such patients have

a. abnormal distributions of thymus processed (T), bursal equivalent (B) and null peripheral lymphocytes b. anti-lymphocyte antibodies occur in association with increased disease activity. These antibodies are not specific for particular

classes of lymphocytes.

B. Blood lymphocytes from some patients with Sjogren's Syndrome have been previously shown to have multiple heavy chain determinants on their surface. New studies (J. van Boxel, S. Paget) revealed that these are due to auto anti-lymphocyte antibodies.

C. The biochemical lesions in the genetic mucopolysaccharidoses and mucolipidoses are under intensive investigation (E. F. Neufeld, P. Di Natale, L. Rome, C. Hall, J. F. Scott, I. Liebaers, P. Dillon, G. Sando, I. Leder). The major new findings are:

1. The kinetics of uptake of α-L-iduronidase into Hurler fibroblasts are compatible with the model of a cell-surface receptor interacting with a "recognition marker" on the enzyme. Some sugars and glycosides inhibit the uptake, but the most potent competitive inhibitor is found in a glycoprotein fraction from urine.

2. α-L-iduronidase has been purified from human kidneys about 10,000 fold, and is nearly homogeneous. This purified enzyme was injected into goats and a high titer antiserum to iduronidase was obtained.

3. A new assay for α-L-iduronidase has been devised to relieve the shortage of the substrate currently used, phenyliduronide. The new assay is based on the hydrolysis of iduronosyl-anhydromannitol [^{3}H] (prepared by Dr. I. Leder, LBM) and the separation of the neutral radioactive product from the charged substrate on small ion exchange columns.

4. Iduronate sulfatase activity in amniotic fluid was shown to be diagnostic of the Hunter syndrome in the fetus. A carrier test, based on measurement of iduronate sulfatase activity in lymphocytes, has been evaluated and shown to detect only half of the carriers of the Hunter syndrome.

5. Although the Hunter syndrome is X-linked and thought to occur only in males, we have diagnosed the disease in two girls. Available evidence suggests that there may be an autosomal recessive form of that disease.

6. The chromosomal localization of a human gene encoding the lysosomal enzyme, arylsulfatase A, is mapped with the use of somatic cell hybrids supplied by Drs. J. F. Conscience and F. Ruddle of the Biology Department of Yale University. Chromosomes 12, 3 and 21 are presently likely candidates, all other chromosomes having been excluded.

Practical applications derived from these studies include: improved diagnostic tests, improved ability for genetic counseling, and increasing likelihood that enzymatic replacement therapy can be developed.

III. Disease Mechanisms - Animal Models

A. The New Zealand mouse analogue of human systemic lupus erythematosis continues to be investigated intensively (A. D. Steinberg, N. L. Gerber, L. Klassen, G. Williams, J. P. Reeves, M. Gelfand). New findings include: 1) Abnormal age-related changes in graft vs. host reactions of

NZB/W spleen cells. Changes in suppressor T cells are implicated. 2) Evidence that the anti-T cell antibodies spontaneously arising in NZB/NZW mice preferentially recognize (and eliminate) suppressor cells. 3) Direct correlations between the presence of virus infection and development of autoimmunity have been observed in athymic "nude" mice bred on infected or non-infected "backround" strains.

Additional studies (A. D. Steinberg, D. Eilat, M. Akizuki, I. Scher, E. S. Raveche, J. P. Reeves) on the genetics of autoantibody production in mice reveal a correlation between responsiveness to nucleotide antigens and propensity to autoantibody production which appears to be X-chromosome linked.

The mouse model is useful for developing new therapeutic regimens. Recent studies (A. D. Steinberg, N. L. Gerber, L. Klassen, G. Williams, D. Budman, J. P. Reeves) revealed good responses to a number of immunosuppresive drugs if these were given before the development or at an early stage of the disease. Vibrivin (virazole) an antiviral agent reduced the severity of the autoimmune process. Grafting of thymuses from young mice reduced the severity of several disease parameters.

B. Autoimmune thyroditis on guinea pigs provides another useful model of human autoimmunity. The lymphocytes infiltrating affected areas have been characterized (J. van Boxel, S. A. Paget).

C. Mycoplasma hyorhinis arthritis of swine provides an interesting animal model of a chronic arthritis which has been intensively investigated by J. L. Decker, R. G. Aptekar and L. Barden. Conclusions from these studies are

1. There is an arthrotropic mycoplasma whose effect upon swine depends in part upon host species and in part upon route of administration.

2. Several months after infection the organism cannot be grown out but the arthritis persists for at least 15 months thereafter. There is local antibody production which declines with time.

3. An antigen, typical of the original organism, can be identified in decreasing amounts to its virtual loss by month 18.

4. Skin test inoculation of lifeless antigen makes the host sick-he is in a sustained state of hypersensitivity.

5. In teleological terms, the persistent and destructive synovitis appears to represent the animals' attempt to eliminate what is probably then a non-viable antigen.

IV. Fundamental Studies

A. Cell surface markers continue to be useful for identifying subclasses of lymphocytes. The function of such markers are under continuing investigation. Fc receptors on T-lymphocytes appear when these cells are activated (J. A. van Boxel, D. L. Rosenstreich) though which subclass of T-cells remains to be determined. IgD on B lymphocytes of cord blood appears

to be often in association with both IgG and IgM and its presence in cells of various species is being assessed (J. A. van Boxel, F. D. Funkelman, R. Asofsky, W. E. Paul). Of particular interest is the possible role of IgD in regulating B-cell responsiveness a subject being intensively studied by following Ig synthesis in cells treated with anti-IgD (J. A. van Boxel, M. R. Blaese, S. Broder, T. A. Waldman).

B. The study of the removal of bilirubin and other albumin-bound substances in the blood via extracorporeal perfussion through albumin-agarose beads is continuing (P. H. Plotz, N. Catterall, P. Berk, B. Blitzor, J. Waggoner). The method has been found useful in animal model studies and its applicability to a variety of scavenging procedures is under study.

C. Rheumatoid factor is almost a sine qua non of rheumatoid arthritis. Its specificity has continued to be questioned. Recent work (R. Eisenberg, P. H. Plotz) strongly suggests that the antigenic determinant is on normal IgG and that it is unnecessary to postulate a new determinant arising as a consequence of the disease process.

D. Much of the pathology in autoimmune diseases arises secondary to the formation of immune complexes. The production of stable complexes via bivalent affinity labeling reagents (P. H. Plotz) may be useful in the study of how these complexes 'work'.

E. Antigen-induced IgE-mediated mast cell degranulation is an important component of allergic reactions and inflammatory mechanisms and serves as a useful analogue for antigen induced antibody-mediated reactions in general. This system is being explored in depth (H. Metzger, C. Isersky, G. Mendoza, S. Newman, G. Rossi). Major new developments include 1) isolation of full active membrane fragments and their characterization 2) solubilization of soluble receptors and their characterization 3) elicitation of anti-receptor antibodies which can be used to probe the mechanism of receptor triggering. Studies to determine the site(s) on IgE which interact with the receptor may ultimately provide an approach to learning now to block the binding thereby providing a possible therapeutic approach.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41000-07 ARB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Controlled study of cyclophosphamide and azathioprine in systemic lupus erythematosus with nephritis

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

PI:	J.L. Decker	Chief, Arth. & Rheum. Branch	ARB	NIAMDD
	A.D. Steinberg	Senior Investigator	ARB	NIAMDD
OTHER:	P.H. Plotz	Senior Investigator	ARB	NIAMDD
	P. Rooney	Guest Worker	ARB	NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Arthritis & Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/4	1	1/4

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

Patients with diffuse glomerulonephritis and systemic lupus erythematosus satisfying criteria for active renal disease are offered a therapeutic study. The patients are randomized to different treatment programs which include the following drugs: corticosteroids, azathioprine and cyclophosphamide.

Follow-up visits occur every six months and the patients are followed until death or dialysis. Clinical and immunologic assessment are performed. The risks and benefits of each treatment will be evaluated and compared.

PHS-6040
(12-75)

Project Description:

Objectives:

Two previous ten week studies by our branch suggested that cyclophosphamide was effective in the treatment of lupus nephritis and that azathioprine was less effective. A long-term study of these patients is being carried out to determine whether or not one therapeutic program is superior over the course of several years. In addition, newer drug programs have been suggested by study in New Zealand mice.

Methods Employed:

Patients meeting predefined criteria for diagnosis of SLE and activity of nephritis have been admitted to the trial. In a baseline control period, the patients were kept on the anti-inflammatory regimen (usually prednisone and aspirin) needed to control extra-renal manifestations. These have been continued into the study period with as little change as possible and with the addition of randomly assigned 1) cyclophosphamide, 2) azathioprine, 3) cyclophosphamide plus azathioprine, 4) intravenous cyclophosphamide and 5) steroids only.

A large number of serological, chemical and urine values are serially obtained and the patients will be followed for life. Immunolglobulin levels, anti-DNA antibodies, and serum complement are among those measures. We will determine time to death or renal dialysis in each treatment program and assess the toxicity as well as efficacy of each program.

Major Findings:

Patients receiving no immunosuppressive drug have progressed to death or dialysis at a rate somewhat faster than those receiving drug. However, there has been no significant differences among the programs. Toxicity (real and potential) from cyclophosphamide and azathioprine given separately in the absence of major long-term advantages over 4 years of follow-up suggest that these programs be discontinued in the future.

Significance to Bio-Medical Research and the Program of the Institute:

If difference in the response of the disease and some of the immunological processes can be detected between different drug programs, it will be possible to draw conclusions with reference to the cell populations involved in systemic lupus erythematosus and thus increase our understanding of the disease process itself. It is expected that the results will permit us to select among treatment programs in clinical management.

Proposed Course:

A long-term follow up of these patients will be continued, primarily by one week admissions every 6 months. Since programs 1 and 2 have not been convincingly effective, new patients will not be admitted to them. New patients will continue to be admitted to the study groups 3, 4 and 5.

Publications:

Decker, J.L., Klippel, J.H., Plotz, P.H. and Steinberg, A.D.: Cyclophosphamide or azathioprine in the treatment of lupus glomerulonephritis. A controlled trial - results at 28 months. Ann Intern. Med. 83:606-615, 1975.

Decker, J.L. and Steinberg, A.D.: The current status of cytotoxic drug therapy in rheumatoid arthritis and systemic lupus erythematosus. (In Russian) Ter. Arh., 1976. (In press)

Gerber, N.L. and Steinberg, A.D.: Clinical use of immunosuppressive drugs. Part I. Drugs 11:36-44, 1976.

Steinberg, A.D.: Clinical use of immunoregulants. Rosenthale, M.E. and Mansmann, H.C. (Eds.). Immunopharmacology. New York, Spectrum Pub., 1976, pp. 267-274.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41001-05 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Therapy of SLE: A controlled trial comparing high dose intravenous cyclophosphamide with placebo

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

PI:	P. H. Plotz	Senior Investigator	ARB NIAMDD
OTHER:	J. L. Decker	Chief, Arthritis & Rheumatism Branch	ARB NIAMDD
	A. D. Steinberg	Senior Investigator	ARB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Connective Tissue Disease

SECTION
Arthritis & Rheumatism Branch

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	3/4	1/4

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

At present, no satisfactory therapy exists for severe manifestations 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 doses of prednisone (up to 2 mg per kg for 3 weeks).

PHS-6040
(12-75)

Project Description:

Objectives:

At present no satisfactory therapy exists for severe manifestations 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 a 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:

Six patients have been admitted to the study so far - two in the non-renal group and four 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.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41002-11 ARB

PERIOD COVERED July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Retrospective Evaluation of Knee Synovectomy in Rheumatoid Arthritis

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

PI:	J. L. Decker	Chief, Arthritis and Rheumatism Branch	ARB	NIAMDD
OTHER:	R. G. Aptekar	Clinical Associate	ARB	NIAMDD
	L. T. Peterson	Consultant, Geo. Washington Univ. Sch.Med.		
	D. Fried	Chief, CC Rehabilitation Dept.	Rehab	CC

COOPERATING UNITS (if any)

George Washington University School of Medicine and Rehabilitation Department, CC

LAB/BRANCH

Arthritis & Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

This project has been delayed due to the sabbatical of the Principal Investigator

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41003-03 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Synovial changes in rheumatoid arthritis under cyclophosphamide therapy.

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

PI:	J. L. Decker	Chief, Arthritis & Rheumatism Branch	ARB	NIAMDD
OTHER:	R. G. Aptekar	Clinical Associate	ARB	NIAMDD
	H. Cattell	Consultant, Geo. Wash. Univ. Hosp.		
	Hope Hopps	Asst. to Dir., BB	BB	DIR

COOPERATING UNITS (if any)
George Washington University Hospital and Bureau of Biologics, DIR

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

This project has been delayed due to the sabbatical of the Principal Investigator.

OHS 6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41004-11 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Role of infection in rheumatoid arthritis

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

PI:	J. L. Decker	Chief, Arthritis & Rheumatism Branch	ARB	NIAMDD
OTHER:	R. G. Aptekar	Clinical Associate	ARB	NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

This project has been completed. Work is being done on a fifth manuscript.

Decker, J. L. and Barden, J. A. Mycoplasma hyorhinis Arthritis of Swine: A Model for Rheumatoid Arthritis? Rheumatology 6:338-345, 1975.

Manuscript in preparation.

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41005-04 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Cellular and humoral immunity in systemic lupus erythematosus (SLE) undergoing randomized cytotoxic therapy

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

PI:	J. L. Decker	Chief, Arthritis & Rheumatism Branch	ARB	NIAMDD
OTHER:	A. D. Steinberg	Senior Investigator	ARB	NIAMDD
	R. G. Aptekar	Clinical Associate	ARB	NIAMDD
	T. A. Waldmann	Chief, Metabolism Branch	MET	NCI

COOPERATING UNITS (if any)
Metabolism Branch, NCI

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

This study has been completed and a manuscript is in preparation.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41006-03 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Characterization of infiltrating lymphocytes in rheumatoid synovial membranes

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

PI:	J. A. van Boxel	Visiting Scientist	ARB NIAMDD
OTHER:	D. Torretti	Clinical Associate	ARB NIAMDD
	J. L. Decker	Chief, Arthritis & Rheum. Br.	ARB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	3/4	1/4

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

Immunological mechanisms in the pathogenesis of rheumatoid arthritis may be revealed by study of the remarkably dense lymphocytic infiltration in rheumatoid synovial membranes. A method was developed for extraction of viable intralesional lymphocytes by digestion of synovium with collagenase and deoxyribonucleases and B and T cells quantitated in these populations using surface markers of these cells. Adherence of C sensitized erythrocytes to C3 bearing B cells in cryostat tissue sections were also examined. The infiltrate was found to consist predominantly of T lymphocytes. Attempts will be made to assess the functional potential of these lymphocytes. Cytotoxic potential of the intralesional lymphocytes as well as their sensitization to tissue antigens will be investigated in tissue culture.

PHS-6040
(12-75)

Project Description:

Objectives:

Immunological mechanisms in the pathogenesis of rheumatoid arthritis may be revealed by study of the remarkably dense lymphocytic infiltrations in rheumatoid synovial membranes. We have determined by B and T lymphocyte compositions of this infiltrate and will attempt to assess some of the functional capacities of these intralesional lymphocytes. A major question is whether the lymphocytes are engaged in an auto-destructive process.

Methods Employed:

A method was developed for the extraction of viable lymphocytes from the synovium using a digestion procedure. B and T cells were quantitated in suspension and by adherence of indicator cells to cryostat sections.

Major Findings:

T cells were found to predominate while B cells accounted for most of the remainder. True germinal centers were shown to occur in the synovium. The extraction method has general applicability.

Significance to Bio-Medical Research and the Program of the Institute:

There is much evidence for immunological mechanisms having a role in the pathogenesis of rheumatoid arthritis. Since B and T cells form functionally as well as ontogenetically distinct populations, study of intralesional B and T cells may provide information concerning pathogenetic mechanisms. Striking improvement has been reported as a result of thoracic duct drainage, a procedure depleting primarily T cells; is not inconceivable that this maneuver is effective because it depletes synovial T cells, which have been shown to have the capacity to destroy target cells *in vitro*.

Proposed Course:

Attempts will be made to assess the cytotoxic capacities of these isolated intralesional lymphocytes in tissue culture. Both T cell cytotoxicity and antibody dependent lymphocyte mediated (non-T-cell) cytotoxicity will be assessed using fibroblasts and chondrocytes as target cells and peripheral and intralesional lymphocytes from patients as effectors. Antigen induced lymphocyte transformation between these cell types and using cell free extracts of synovium will also be tested.

Publications:

1. van Boxel, J.A. and Paget, S.A.: Predominantly T-cell infiltrate in rheumatoid synovial membranes. *New Eng. J. Med.* 293:517-520, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41007-02 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Cell surface immunoglobulins of lymphocytes in Sjogrens syndrome

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

PI:	J. A. van Boxel	Visiting Scientist	ARB NIAMDD
OTHER:	S. A. Paget	Clinical Associate	ARB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	1/2	1/4

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

Multiple heavy chain determinants have previously been shown on blood lymphocytes of some patients with Sjogren's syndrome. We will investigate the mechanism accounting for this phenomenon, particularly the possible role of anti-lymphocyte antibodies by incubating normal cells in serum from such patients, to determine if such cells acquire these determinants.

This project has been completed and the manuscript is in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41008-02 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Characterization of infiltrating lymphocytes in experimental autoimmune thyroiditis of the guinea pig

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

PI:	J. A. van Boxel	Visiting Scientist	ARB NIAMDD
OTHER:	S. A. Paget	Clinical Associate	ARB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	1/2	1/4

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

Experimental autoimmune thyroiditis will be used as a model for studying the B and T cell composition of the dense lymphocytic infiltrate, a lesion considered to be a cell-mediated immunological reaction. Glands will be digested with collagenase to provide suspensions of cells. Standard procedures will be used to assess B and T cell markers. Functional parameters of infiltrating cells will be assessed.

PHS-6040
(12-75)

Project Descripton:

No further experiments will be done on this project. A manuscript is in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41009-02 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies of IgD on cord blood lymphocytes and mouse lymphocytes

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

PI:	J. A. van Boxel	Visiting Scientist	ARB NIAMDD
OTHER:	F. D. Finkelman	Research Associate	LI NIAID
	R. Asofsky	Chief, Lab. Microbial Immunity	LMI NIAID
	W. E. Paul	Chief, Lab. Immunology	LI NIAID

COOPERATING UNITS (if any)
Laboratory of Immunology and Laboratory of Microbial Immunity, NIAID

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/4	1	1/4

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

Cell membrane IgD is to be analyzed by 1) Cocapping studies using antibodies coupled to different fluorochromes, in cord blood and mouse lymphocytes for possible presence of IgD. 2) Surface iodination techniques in which Ig is extracted with a non-ionic detergent, precipitated with anti-Ig serum and electrophoresed on SDS polyacrylamide gels. A peak of the mobility corresponding to "IgD" will be looked for in mouse, rabbit, guinea pig and compared with cord blood.

PHS-6040
(12-75)

Project Description:

Objectives:

IgD appears to be the major surface immunoglobulin on neonatal (cord blood) lymphocytes. This fact may point to a biological role for this molecule; in addition the high proportion of IgD bearing lymphocytes facilitates study of these cells. Efforts to establish a function for cell-membrane IgD have been hampered by the lack of animal models, as IgD has only been identified in man.

Methods Employed:

Cell membrane Ig was analyzed by 1) cocapping studies using antibodies coupled to different fluorochromes (rhodamine and fluorescein). 2) Surface iodination techniques in which Ig is extracted with a non-ionic detergent, precipitated with anti-Ig serum and electrophoresed on SDS polyacrylamide gels.

Major Findings:

1. A high frequency of IgD bearing cells in some specimens of cord blood appear to bear IgG in addition to IgM.

2. Recently an Ig class on mouse lymphocytes has been found by surface iodination techniques which has been proposed as a homologue of IgD. Iodination and extraction procedures followed by gel electrophoresis confirmed the presence of an H chain with mobility more rapid than that of μ chain (putative IgD), although this was not found in rabbit or guinea pig. Delta chain identified on cord blood lymphocytes had a mobility similar to human and mouse μ chain, however.

Significance to Bio-Medical Research and the Program of the Institute:

The presence of IgG on IgD bearing cord blood lymphocytes if confirmed is contrary to the current concept of IgD sharing the cell membrane exclusively with IgM. Evidence is presented that appears to support the presence of an IgD homologue in the mouse, using immunofluorescence and surface iodination procedures.

Proposed Course:

Studies will be directed to further elucidate the biological role of IgD.

Publications:

Finkelman, F.D., van Boxel, J. A., Asofsky, R., and Paul, W. E.: Cell membrane IgD. Demonstration of IgD on human cord blood lymphocytes by enzyme catalyzed iodination and comparison with cell surface Ig of mouse, guinea pig and rabbit. J. Immunol. 116:1173-1181, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41010-02 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Effect of purified antibody to IgD on lymphocyte transformation and immunoglobulin production.

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

PI:	J. A. van Boxel	Visiting Scientist	ARB	NIAMDD
OTHER:	M. R. Blaese	Head, Cellular Immunology Section	MET	NCI
	S. Broder	Clinical Associate	MET	NCI
	T. A. Waldman	Chief, Metabolism Branch	MET	NCI

COOPERATING UNITS (if any)
Metabolism Branch, NCI

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/4	1	1/4

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

The effect of purified antibody to IgD was tested for effects on in vitro immunoglobulin synthesis by human blood lymphocytes. Inhibition of Ig production was found at low concentrations of anti-IgD. The Fc fragment of the antibody did not have a role in this suppression.

PHS-6040
(12-75)

Project Description:

Objectives:

We were previously the first to demonstrate that IgD, an immunoglobulin class of as yet unknown function, is bound to the surface membrane of an unexpectedly high frequency of human lymphocytes. So as to investigate the biological role of IgD the effects on purified antibody to IgD was tested in various *in vitro* systems for possible stimulator or inhibitor effects.

Methods Employed:

IgD myeloma protein was purified using DEAE cellulose chromatography followed by G 200 gel filtration. Goat antibody to IgD was purified by affinity chromatography. Anti-IgD was added to cultures of human lymphocytes and to the mixed lymphocyte reaction and effects monitored by standard methods of isotope incorporation. Ig secretion was measured by radioimmunoassay.

Major Findings:

A striking inhibition of the *in vitro* synthesis of the three major serum heavy chain classes was found to occur when anti-IgD was added to cultures of human peripheral blood lymphocytes. This inhibition was seen at very low levels of added antibody. Inhibitory effects were found to be proportional to the amount of anti-IgD added. $F(ab^1)_2$ fragments of anti-IgD were equally suppressive indicating that the effect was not mediated via the Fc portion of the Ig molecule.

Significance to Bio-Medical Research and the Program of the Institute:

IgD is currently the focus of a great deal of research interest. It is probably the major cell surface immunoglobulin in mammals. However, concepts as to its role in the immune system are as yet entirely speculative. Although the significance of anti-IgD inhibition of Ig synthesis *in vitro* are not clear at this stage, it may have implications with regard to a receptor function of cell surface IgD.

Proposed Course:

Investigations will be directed at elucidating the significance of the *in vitro* inhibitor effect of anti-IgD.

Publications:

1. van Boxel, J. A., Broder, S. and Waldmann, T. A.: Inhibition of immunoglobulin synthesis *in vitro* by purified antibody to IgD. In Eisvoogel, E. (Ed.): Proceedings of the 10th Annual Leukocyte Culture Conference. Amsterdam, North Holland Publishing Corporation, 1976.

Publications (cont'd)

2. Manuscript in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41011-03 ARB

PERIOD COVERED July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Binding of aggregated γ-globulin to T-lymphocytes

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

PI:	John A. van Boxel	Visiting Scientist	ARB	NIAMDD
OTHER:	David L. Rosenstreich	Sr. Surgeon	LMI	NIDR

COOPERATING UNITS (if any)

Laboratory of Microbiology and Immunology, NIDR

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/2	0

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

Efforts are being directed towards establishing that Fc receptors appear on activated T cells in man. Lymphocytes will be activated in vitro by the mixed lymphocyte reactions.

PHS-6040

Project Description:

Objectives:

We have previously shown in the guinea pig that a subpopulation of thymus-derived or T-lymphocytes have the capacity to bind aggregated γ-globulin, a property hitherto believed to be exclusive to bone-marrow derived or B-cells. The findings suggested that T cells developed an Fc receptor upon activation. We aim to extend these findings to determine whether T cells (in man) develop an Fc receptor upon allogeneic stimulation in vitro.

Methods Employed:

Mixed lymphocyte reactions using human peripheral blood lymphocytes were set up using standard procedures. Aggregated guinea pig γ-globulin was used to detect the putative Fc receptor and simultaneous sheep cell rosettes used as a marker for T cells.

Major Findings:

Preliminary findings indicate that allogeneically stimulated human T cells develop the capacity to binding aggregated γ-globulin in vitro and hence presumably acquire Fc receptors.

Significance to Bio-Medical Research and the Program of the Institute:

Fc receptors on T cells may well play a major role in basic immunological reactions such as for example antigen processing. Furthermore, reports of the existence of T cell immunoglobulin may be explained by adherence of exogenous Ig via this receptor. The finding of T cells with apparent Fc receptors in the MLC reaction may extend these findings to an in vitro model in man.

Proposed Course:

Using the model systems described we will attempt to provide answers to the questions (1) do Fc receptor bearing T cells fall into one of the recently described functional categories of T cells--helper, suppressor, cytotoxic (2) do anti Ia antisera block T cell Fc receptors as has been shown for B cells.

Publications:

Manuscript in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41015-02 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Therapeutic studies in psoriatic arthritis

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

PI:	P. H. Plotz	Senior Investigator	ARB	NIAMDD
OTHER:	A. D. Steinberg	Senior Investigator	ARB	NIAMDD
	E. Milstone	Guest Worker	OD	CC
	R. Black	Assoc. Dir., CC	OD	CC
	N. L. Gerber	Guest Worker	ARB	NIAMDD
	P. Lucky	Clinical Associate	ARB	NIAMDD
	S. A. Paget	Clinical Associate	ARB	NIAMD

COOPERATING UNITS (if any)
Office of the Director, CC

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

This project was never activated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41016-04 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The removal of bilirubin and other albumin bound substances from blood

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

PI:	P. H. Plotz	Senior Investigator	ARB NIAMDD
OTHER:	N. Catterall	Technician	ARB NIAMDD
	P. Berk	Chief, Section on Diseases of the Liver	DD NIAMDD
	B. Blitzer	Clinical Associate	DD NIAMDD
	J. Waggoner	Chemist	DD NIAMDD

COOPERATING UNITS (if any)
Digestive Diseases Branch, NIAMDD

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/4	3/4	1/2

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

Previous studies have established the feasibility of removing albumin-bound substances such as bilirubin from the blood by in vivo extracorporeal perfusion using albumin-agarose beads. Because a number of substances in addition to bilirubin accumulate in the blood of patients with liver damage, we plan to test albumin-agarose beads and other adsorbents in extracorporeal perfusion of experimental animals with liver damage. At present we are setting up the assays to measure blood levels of some substances known to accumulate in liver failure, amino acids, fatty acids, mercaptans, and the false neurotransmitter, octopamine. We will test the efficacy of various adsorbents for binding these substances.

Project Description:

Objectives:

Substances such as bilirubin that bind tightly to plasma protein cannot be removed by dialysis. We have attempted to remove such substances by employing affinity chromatography on albumin-agarose beads.

Methods Employed:

Human albumin is conjugated to agarose: the resulting beads are placed in a column and plasma or whole blood, are passed over the column. Bilirubin or other substances are assayed before and after passage. The bound material is eluted and assayed.

Major Findings:

Agarose-albumin columns can bind in excess of 150 µg bilirubin per gram of wet weight of gel. The BR can be completely eluted and the gel reused. The gel material is compatible with blood and can be used in a perfusion system with living rats and newborn monkeys. Binding of calcium by citrate prevents platelet and white cell losses in the extracorporeal circuit.

Significance to Bio-Medical Research and the Program of the Institute:

Such conjugates may be useful for removing bilirubin and other protein-bound toxic substances from human blood.

Proposed Course:

Further studies will include investigation of the binding of other substances to agarose-protein beads and the preparation of materials safe for *in vitro* use. *In vivo* studies on rats, monkeys and sheep have begun.

Publications:

1. Wolkoff, A. W., Scharschmidt, B. F., Plotz, P. H. and Berk, P. D.: Purification of conjugated bilirubin: A new approach utilizing albumin-agarose gel affinity chromatography. Proc. Soc. Exp. Bio. Med. (In press)

2. Scharschmidt, B. F., Martin, J. F., Shapiro, L. J., Plotz, P. H., and Berk, P. D.: Hemoperfusion through albumin-conjugated agarose gel for the treatment of neonatal jaundice in premature rhesus monkeys. J. Lab. Clin. Med. (In press)

Publications (cont'd):

3. Scharschmidt, B. F., Martin, J. F., Shapiro, L. J., Plotz, P. H., and Berk, P. D.: The use of calcium chelating agents and prostaglandin E_1 to eliminate platelet and white blood cell losses resulting from hemoperfusion through uncoated charcoal, albumin-agarose gel, and neutral and cation exchange resins. J. Lab. Clin. Med. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41017-02 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The valence and specificity of rheumatoid factor

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

PI:	R. Eisenberg	Clinical Associate	ARB	NIAMDD
OTHER:	P. H. Plotz	Senior Investigator	ARB	NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

This project has been completed and led to a publication.

Eisenberg, R.: The specificity and polyvalency of binding of a monoclonal rheumatoid factor. Immunochemistry 13:355-359, 1976.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41018-02 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Production of covalently-crosslinked immune complexes

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

PI: P. H. Plotz Senior Investigator ARB NIAMDD

OTHER: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Connective Tissue Disease

SECTION
Arthritis and Rheumatism Branch

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/4	1/4

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

Purified antibodies will be treated with a reagent capable of covalently-cross-linking the antigen combining sites by affinity-labelling. The complexes will be purified and their properties studied.

PHS-6040
(12-75)

Project Description:

Objectives:

To produce site-specifically covalently cross-linked antigen-antibody complexes to allow study of certain properties of these complexes.

Methods Employed:

Anti-hapten antibodies are cross-linked with a bivalent affinity-labelling hapten.

Major Findings:

Covalently-linked complexes have been produced and are being studied in detail.

Significance to BioMedical Research and the Program of the Institute:

This work may allow studies of the properties of stable immune complexes of various sizes relevant to their role in the pathogenesis of connective tissue disease.

Proposed Course:

Studies will continue.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41020-09 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Pathogenesis of Autoimmunity in New Zealand Mice

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

PI:	A. D. Steinberg	Sr. Investigator	ARB	NIAMDD
OTHER:	N. L. Gerber	Chief, Rehab. Dept.	REHAB	CC
	L. Klassen	Research Assoc.	ARB	NIAMDD
	G. Williams	Clin. Assoc.	ARB	NIAMDD
	J. P. Reeves	Chemist	ARB	NIAMDD
	M. Gelfand	Georgetown Univ. Hospital		

COOPERATING UNITS (if any)

Rehabilitation Department, CC
Georgetown University Hospital

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYLARS:	PROFESSIONAL:	OTHER:
1-1/2	1-1/4	1/4

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

The pathogenesis of autoimmunity in New Zealand mice is still uncertain; however, advances are being made. Genetic, viral and immunologic factors appear to be involved. The immunologic factors are complex. A very early defect is a loss of thymic regulatory or suppressor cells. These cells may keep the immune system in check and when deficient allow autoimmune responses to occur. We are trying to separate and characterize these cells with an aim toward understanding the precise cellular defect.

Antibodies to T cells are produced by New Zealand mice. The role of these antibodies in the loss of suppressor cells is being evaluated. Preliminary experiments indicate that these anti-T cell antibodies preferentially eliminate suppressor cells.

PHS-6040
(12-75)

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 and are under active investigation.

Cellular cooperation between thymus derived (T) cells and bone marrow derived (B) cells is a recently described feature of humoral immunity. Two distinct T cell subpopulations cooperate in cell mediated immunity. The existence of suppressor T cells has been supported by experiments in several systems. We have been investigating such suppressor cell function in New Zealand mice in the hope of understanding the pathogenesis of this autoimmune disease.

Methods Employed:

1. Assay for antibodies to RNA and DNA using ^{14}C-labeled nucleic acids as ligands and the ammonium sulfate precipitation assay. Assay of antibody forming cells to sheep erythrocytes and pneumococcal polysaccharide by local hemolysis in gel.

2. Immunization of animals with a variety of antigens with and without protein carriers or adjuvants.

3. Transfer of spleen, bone marrow and thymus cells into 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 effect in New Zealand mice and other mice.

5. Study of graft versus host disease inducing properties of NZB/W lymphoid cells using the Simonsen spleen weight assay and newborn C3H/He or SWISS mice as recipients, or adult athymic (nude) mice.

6. Separation of lymphoid cells functionally (corticosteroid, ATS, anti-θ, anti-kappa) and physically (sedimentation).

7. Assay of anti-T cell antibodies by release of 51 Cr from thymocytes.

8. Evaluation of subpopulation of T cells using the fluorescence activated cell sorter.

Major Findings:

1. Suppressor cells have been studied in cellular immunity using the graft versus host reaction (GVH) model. The GVH response measured in newborn C3H mice to 5 x 10^6 NZB/W spleen cells increases between 1 and 6 months and then falls to low levels by 12 months. This age change does not occur in control mice. The high response of 4 1/2 to 6 month NZB/W spleen cells was suppressed by thymocytes or spleen cells from young NZB/W mice. Suppressive activity of thymus was lost gradually between 1 and 4 months of age. Thymocytes from 4 month NZB/W mice could synergize with 12 month NZB/W spleen cells, but could not suppress 4 1/2 month spleen cells, whereas thymocytes from 1 month NZB/W mice could synergize with 12 month NZB/W spleen cells and suppress 4 1/2 month spleen cells. Control strains showed neither suppression nor synergy.

2. Fractionation of thymocytes from 1 month old NZB/W mice at unit gravity yielded one fraction enriched in helper cells and another enriched in suppressor cells.

3. Athymic "nude" mice were found to develop spontaneously antibodies to DNA and immune complex glomerulonephritis. These mice were bred on a BLAB/c background and were known to be infected with several viruses. Athymic "nude" mice on a SWISS genetic background and not carrying these viruses did not develop autoimmunity.

4. Fractionated (unit gravity sedimentation) thymocytes from 1 month old NZB/NZW mice have different densities of θ antigen. The surface characteristics of subpopulation of T cells from NZB/W mice of different ages as well as non-autoimmune mice will be studied.

5. Anti-T cell antibodies which occur spontaneously in NZB/NZW mice preferentially recognize and eliminate suppressor cells.

Proposed Course:

Future studies will attempt to further define and separate different subpopulations of T cells, as well as the relative changes in such populations with age (helper, suppressor, effector, etc.) The characteristics of the separated subpopulations will be defined, particularly those of suppressor cells. The role of genetic and viral factors and anti-T cell antibodies in the loss of suppressor cells will be investigated in New Zealand mice. The availability of athymic nude mice will allow us to study lack of thymic function in different strains with and without superinfection with different viruses or immunization with nucleic acid antigens. We hope thereby to define the relative contributions of genetic, viral, and immunologic factors in the development of autoimmunity.

Publications:

1. Steinberg, A. D., Gerber, N. L., Gershwin, M. E., Morton, R., Goodman, D., Chused, T. M., Hardin, J. A. and Berthold, D. R.: Loss of suppressor T cells in the pathogenesis of autoimmunity. In Singhal, S. K. and Sinclair, St. C. (Eds.): Suppressor Cells In Immunity. Ontario, London, Canada, The University of Westum, 1975, pp. 175-180.

2. Gerber, N. L. and Steinberg, A. D.: Physical of "suppressor" from "Helper" thymocytes. J. Immunol. 115: 1744,1745, 1975.

3. Thurman, G. B., Steinberg, A. D., Ahmed, A., Gershwin, M. E. and Goldstein, A. L.: Effects of thymosin treatment in vivo. Increased mitogenic responsivity of murine lymph node lymphocytes. Transplantation Proc. 7 (suppl. 1): 299, 304, 1975.

4. Gershwin, M. E., Steinberg, A. D., Woody, J. N., Ahmed, A.: Studies of thymic factors. I. Evaluation of the mouse rosette assay for thymic hormone. J. Immunol. 115: 1444, 1448, 1975.

5. Gershwin, M. E., Merchant, B., Gelfand, M. C., Vickers, J., Steinberg, A. D. and Hansen, C. T.: The natural history and immunopathology of ourbred athymic (nude) mice. Clin. Immunol. Immunopathol. 4: 324, 340, 1975.

6. Hyman, L. R., Colvin, R. B. and Steinberg, A. D.: Immunogenetics of autoimmune tubulointersititial nephritis. I. Demonstration of differential susceptibility in Strain XIII and Strain II guinea pigs. J. Immunol. 116: 327, 335, 1976.

7. Selgrade, M. K., Ahmed, A., Sell, K. W., Gershwin, M. E. and Steinberg, A. D.: Effect of murine cytomegalovirus on the in vitro responses of T and B cells to mitogens. J. Immunol. 116: 1459, 1465, 1976.

8. Gelfand, M. C., Steinberg, A. D. and Paul, W. E.: Rapid appearance of complement receptor lymphocytes in mice having autoimmune and lymphoproliferative disorders. J. Rheumatology 1 (supplement 1): 50, 1974.

9. Ochiai, T., Scher, I., Ahmed, A., Sell, K. W. and Steinberg, A. D.: Functional characterization of a thymocytotoxic antibody present in NZB mouse serum. J. Rheumatol. 1 (supplement 1): 51, 1974.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41021-08 ARB

PERIOD COVERED: July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Antinuclear antibodies in spontaneous and drug-induced systemic lupus erythematosus and other diseases

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

PI:	A. D. Steinberg	Senior Investigator	ARB NIAMDD
OTHER:	D. Eilat	Visiting Scientist	LCB NIAMDD
	M. Akizuki	Visiting Fellow	ARB NIAMDD
	I. Scher	National Naval Medical Center	NNMC
	E. S. Raveche	Chemist	LEP NIAMDD
	J. P. Reeves	Chemist	ARB NIAMDD

COOPERATING UNITS (if any)
Laboratory of Chemical Biology, NIAMDD
Laboratory of Experimental Pathology, NIAMDD
National Naval Medical Center, Bethesda, Maryland

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/2	1	1/2

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

Antibodies to nucleic acids are important diagnostically and pathogenetically in systemic lupus. The production of these antibodies is in part controlled by x-linked immune response genes. In addition, sex hormones may influence the magnitude of auto-antibody production. Antibodies to transfer RNA are produced by patients with SLE; these cross react with viral nucleic acids.

PHS-6040
(12-75)

Project Description:

Objectives:

Study the characteristics of a variety of antinuclear antibodies in human and murine "autoimmune diseases." Determine genetic contributions to the immune response to nucleic acid and related antigens.

Methods Employed:

Farr ammonium sulfate precipitation assay using radiolabelled ligands. Standard precipitin reaction using labelled and unlabelled nucleic acids or nuclear extracts. Hemagglutination, complement fixation and immunodiffusion assays. Standard immunization procedures.

Major Findings:

1. Some strains of mice (NZB and ALN) were found to have high responses to immunization with single stranded DNA complexed to a carrier protein. These animals also spontaneously develop antinuclear antibodies late in life. In contrast DBA/2 and BALB/C mice responded poorly to immunization with SSDNA. These mice do not spontaneously develop antinuclear antibodies. Genetic studies of NZB X DBA F_1, mice and backcrosses to the parental strains suggest that a gene on the X chromosome may influence the response to immunization with SS DNA.

2. Castration of male NZB x DBA or DBA x NZB mice led to accelerated autoantibody production.

3. The immune response to several thymic independent antigens is influenced by gene (Ir) on the X chromosome.

4. NZB/NZW mice and patients with SLE spontaneously produce antibodies to transfer RNA.

Significance to Bio-Medical Research and the Program of the Institute:

X-linked immune response genes help to control the immune responses to nucleic acid antigens. Male sex hormones appear to suppress autoantibody production explaining the more severe disease and greater incidence in females.

Proposed Course:

Define the genetics of the antibody response to DNA. Further study the characteristics of antinuclear antibodies. Continue to evaluate the role of sex hormones in autoimmunity. Compare antinuclear antibodies of patients with different autoimmune diseases (SLE, Sjogren's syndrome, rheumatoid arthritis).

Publications:

1. Scher, I., Steinberg, A. D., Berning, A. K., and Paul, W. E.: X-linked B-lymphocyte immune defect in CBA/N mice. II. Studies of mechanism underlying the immune defect. J. Exp. Med. 142:637-650, 1975.

2. Scher, I., Ahmed, A., Sharrow, S. O., Steinberg, A. D. and Paul, W. E.: Genetic control of B-lymphocyte function in the CBA/N mouse strain: A model for examining the mechanism of B-lymphocyte function in the CBA/N mouse strain: A model for examining the mechanism of B-lymphocyte activation. In Oppenheim, J. J. and Rosenstreich, D. (Eds.): Role of Mitogens in Immunobiology. New York, Academic Press (In press).

3. Eilat, D., Schechter, A. N., and Steinberg, A. D.: Antibodies to native RNA in NZB/NZW mice. Nature 259:141-143, 1976.

4. Gershwin, M. E., Glinski, W., Bender, A. N., Ringel, S. P., Steinberg, A.D. and Engel, W. K.: Antibodies to nucleic acids in myastenia gravis. Int. Arch. Allergy and Appl. Immunol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41022-05 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Therapeutic studies in New Zealand mice

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

PI:	A. D. Steinberg	Sr. Investigator	ARB	NIAMDD
OTHER:	N. L. Gerber	Chief, Rehab. Dept.	REHAB	CC
	L. Klassen	Research Assoc.	ARB	NIAMDD
	G. Williams	Clin. Assoc.	ARB	NIAMDD
	D. Budman	Clin. Assoc.	ARB	NIAMDD
	J. P. Reeves	Chemist	ARB	NIAMDD

COOPERATING UNITS (if any)
Rehabilitation Department, CC

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/2	1-1/4	1/4

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

Non-specific immunosuppressive therapy of man is associated with considerable morbidity. Some regimens are superior to others. The New Zealand mice offer a convenient model for studying drug comparisons prior to study in patients. It would be even more desirable to treat patients in an immunologically more specific manner. Toward that end we have been treating New Zealand mice with activators of suppressor cells and with suppressor cells themselves. Preliminary results with NZB mice suggest that the autoimmune process can be markedly retarded by such specific immunosuppression. These studies will be pursued and an attempt will be made to obtain a soluble suppressor substance so that the technique might be applicable to man.

PHS-6040
(12-75)

Project Description:

Objectives:

NZB/NZW F_1 mice spontaneously develop antibodies to nucleic acids and die of immune complex glomerulonephritis. NZB mice develop Coombs' positive hemolytic anemia, membranous nephrosis and excessive lymphoreticular proliferation. These mice are an excellent model for comparing different immunosuppressive and anti-viral 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. Comparison of different immunosuppressive and anti-viral drug regimens in natural history of NZB/NZW mice - antibodies to DNA, proteinuria and survival.

2. Study of thymus grafting and thymic hormone in an attempt at restoring normal regular functions and improve the natural history of New Zealand mice.

Major Findings:

New Zealand mice responded to a number of immunosuppressive drug regimens when started at 5 months of age or later in life if the animals did not have significant proteinuria. When they had significant proteinuria they did not respond favorably to the immunosuppressive regimens that were effective earlier in life. The favorable response to therapy was associated with reduced deposition of immune complexes in the glomeruli without a reduction in serum immunoglobulin levels.

Chlorambucil, an alkylating agent like cyclophosphamide but without the latter's bladder toxicity appears less effective than cyclophosphamide in treatment of disease in NZB/W mice.

Intermittent (every 2 weeks) grafting of thymuses from young NZB mice to syngeneic NZB mice starting at 1 month of age prevented the development of anti-erythrocyte antibodies and reduced lymphoid proliferation and severity of renal disease. The relevant thymocytes are corticosteroid and X-irradiation sensitive.

Of several anti-viral agents used to treat NZB/NZW mice, vibarivin (virazole) reduced the severity of the autoimmune process.

Significance to Bio-Medical Research and the Program of the Institute:

These studies are designed to provide background experience for clinical studies in which the immunosuppressive drugs may be given in a more effective manner to humans with SLE. Trials of anti-viral drugs may be considered in humans at a later time.

Continued study in mice may provide the background information necessary for immunological intervention in humans with SLE.

Proposed Course:

Further study of anti-viral agents. Attempt to treat New Zealand mice with a soluble suppressor substance from suppressor cells.

Publications:

1. Steinberg, A. D., Gershwin, M. E., Gerber, N. L., Hardin, J. A., Barthold, D., Parker, L. M. and Chused, T. M.: Role of suppressor T cells in the pathogenesis of autoimmunity in New Zealand mice. In Siskind, G. W., Litwin, S. and Christian, C. (Eds.): Immune Depression and Cancer. Proceedings of the second Irwin Strasburger Seminar on Immunology. New York, Grune and Stratton, 1975, pp. 42-62.

2. Gelfand, M.C., Schur, P. H., Asofsky, R., and Steinberg, A.D. Therapeutic studies in NZB/NZW mice. IV. Effect of combination drug therapy on immune complex deposition. Arthritis Rheum. 19:43-48, 1976

3. Morton, R. O., Goodman, D. G., Gershwin, M. E., Derkay, C., Squire, R. A., and Steinberg, A. D.: Suppression of autoimmunity and lymphoid proliferation in NZB mice with steroid-sensitive X-radiation sensitive syngeneic young thymocytes. Arthritis Rheum. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41023- ARB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Study of Immune Abnormalities in Patients with Systemic Lupus Erythematosus (SLE)

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

PI:	A. D. Steinberg	Senior Investigator	ARB	NIAMDD
OTHER:	D. Budman	Clinical Associate	ARB	NIAMDD
	L. Klassen	Research Associate	ARB	NIAMDD
	J. P. Reeves	Chemist	ARB	NIAMDD
	W. Glinski	Guest Worker	DB	NCI
	C. Brady	Indian Health Service		
	E. B. Merchant	Dir. Immunol. Hemat. Branch	DBBP	BB

COOPERATING UNITS (if any)

Dermatology Branch, NCI
Indian Health Service
Bureau of Biologics

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-3/4	1-1/2	1/4

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

Particular groups of American Indians have increased annual incidences of SLE. These include Sioux, Crow and Arapaho tribes.

Patients with SLE have anti T cell antibodies and loss of a subpopulation of T cells. This subpopulation may regulate B cell activity. In the absence of adequate regulatory function, excessive antibody forming cells are found. These cells have specificity for a variety of chemical haptens rather than just auto antigen.

PHS-6040
(12-75)

Project Description:

Objectives:

To apply the newer understanding of basic immunologic principles and observations in New Zealand mice (who develop a lupus-like disease) to the study of patients with SLE.

Methods Employed:

1. Study of families of patients with SLE or larger groups of patients with a high incidence of SLE. A battery of immunologic measurements would be made in such patients.

2. Study of lymphocytes from patients with SLE.

a. Distribution on discontinous gradient.

b. Number and distribution of T, B and null cells and their responses to mitogens or allogenic cells.

c. Relationship between anti-lymphocyte antibodies in patients with SLE and abnormalties of cell number and/or function.

d. Study of antibody producing plague forming cells (PFC) in the peripheral blood of patients.

e. Study of anti-lymphocyte antibodies by use of the fluorescence activated cell sorter (FACS).

Major Findings:

1. Particular groups of American Indians have increased annual incidences of SLE. These include Sioux, Crow and Arapaho tribes.

2. Patients with SLE have abnormal distributions of peripheral blood lymphocytes. Those with active disease have a relative decrease in a subpopulation of T cells and an increase in null cells.

3. Anti-lymphocytes antibodies occur in association with increased activity of patients with SLE. These antibodies can reduce the number of perpheral T cells and interfere with their function.

4. Antibodies to human lymphocytes found in sera from patients with SLE are readily studied on the FACS. IgM antibodies to T cells recognize 100% of the T cells of normals and patients with SLE, indicating no specificity for a subpopulation - such as helper or suppressor cells.

5. Patients with active SLE have increased quantities of PFC to defined chemical haptens. Patients with inactive SLE were not significantly different from controls.

Significance to Bio-Medical Research and the Program of the Institute:

Populations for further study are being defined and techniques refined for continued study of humans with SLE. It is hoped that the rewarding observations in the animal model of SLE, New Zealand mice, can be applied to study of human disease itself. The studies of PFC suggest that patients with SLE have generalized B cell hyperactivity rather than selective B cell hyperactivity towards self-antigens.

Proposed Course:

Continue the studies initiated.

Publications:

1. Gershwin, M.E., Hyman, L.R. and Steinberg, A.D.: The choroid plexus in CNS involvement of SLE. J. Pediatrics 87:588-590 1975.

2. Glinski, W., Gershwin, M.E. and Steinberg, A.D.: Fractionation of cells on a discontinous Ficoll gradient. Study of subpopulations of human T cells. J. Clin. Invest. 57:604-614 , 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41024-09 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The Biochemical Lesions in the Genetic Mucopolysaccharidoses and Mucolipidoses

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

PI:	Elizabeth F. Neufeld	Research Chemist and Section Chief	ARB	NIAMDD
OTHER:	Paola Di Natale	Visiting Associate	ARB	NIAMDD
	Leonard Rome	Chemist	ARB	NIAMDD
	Clara Hall	Research Chemist	ARB	NIAMDD
	James F. Scott	Biologist	ARB	NIAMDD
	Ingeborg Liebaers	Guest Worker	ARB	NIAMDD
	Pauline Dillon	Guest Worker	ARB	NIAMDD
	Gloria Sando	Guest Worker	ARB	NIAMDD
	Irwin Leder	Research Chemist	LBM	NIAMDD

COOPERATING UNITS (if any)
Section on Intermediary Metabolism, LBM

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Human Biochemical Genetics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9	7	2

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

The uptake of a lysosomal enzyme, α-L-iduronidase, into fibroblasts from patients with the Hurler Syndrome, appears mediated by interaction of a component of the enzyme ("recognition marker") with a receptor on the cell surface. The structure of that marker, and its alteration in the genetic mucolipidoses will be determined; identification and purification of the receptor will be initiated.

An antibody to α-L-iduronidase will be used to search for cross-reacting mutant proteins in cells of Hurler and Scheie patients; to trace the path of this enzyme from its site of synthesis to its eventual location in lysosomes; and to examine cell surfaces, by immunofluorescence, for bound enzyme.

Similar studies will be initiated for iduronate sulfatase, the enzyme deficient in the Hunter Syndrome, to determine if each lysosomal enzyme has a unique receptor.

PHS-6040

Project Description:

Objectives:

To study normal lysosomal function as well as the biochemical defects in genetic disorders of such function, as in mucopolysaccharidoses and mucolipidoses, and to use the findings for medical assistance to the patients and their families.

Methods Employed:

The standard methods of cell culture and of enzymology are used. The enzyme α-L-iduronidase is purified from urine by previously published methods, and its uptake into Hurler fibroblasts is examined. The same enzyme is purified to homogeneity from human kidneys and injected into goats for production of antibodies. Similar studies are initiated for a related enzyme, iduronate sulfatase. For diagnostic purposes, lymphocytes, serum, amniotic fluid are used in addition to cultured skin fibroblasts.

Major Findings:

1. The kinetics of uptake of α-L-iduronidase into Hurler fibroblasts are compatible with the model of a cell-surface receptor interacting with a "recognition marker" on the enzyme. Some sugars and glycosides inhibit the uptake, but the most potent competitive inhibitor is found in a glycoprotein fraction from urine.

2. α-L-iduronidase has been purified from human kidneys about 10,000 fold, and is nearly homogeneous. This purified enzyme was injected into goats and a high titer antiserum to iduronidase was obtained.

3. A new assay for α-L-iduronidase has been devised to relieve the shortage of the substrate currently used, phenyliduronide. The new assay is based on the hydrolysis of iduronosyl-anhydromannitol [^{3}H] (prepared by Dr. I. Leder, LBM) and the separation of the neutral radioactive product from the charged substrate on small ion exchange columns.

4. Iduronate sulfatase activity in amniotic fluid was shown to be diagnostic of the Hunter syndrome in the fetus. A carrier test, based on measurement of iduronate sulfatase activity in lymphocytes, has been evaluated and shown to detect only half of the carriers of the Hunter syndrome.

5. Although the Hunter syndrome is X-linked and thought to occur only in males, we have diagnosed the disease in two girls. Available evidence suggests that there may be an autosomal recessive form of that disease.

6. The chromosomal localization of a human gene encoding the lysosomal enzyme, arylsulfatase A, is mapped with the use of somatic cell hybrids supplied by Drs. J.F. Conscience and F. Ruddle of the Biology Department of Yale University. Chromosomes 12,3 and 21 are presently likely candidates, all other chromosomes having been excluded.

Significance to Bio-Medical Research and the Program of the Institute:

A number of practical applications have emerged from these studies: improved diagnostic tests; rapid prenatal diagnosis; improved genetic counseling for mucopolysaccharidosis families. Hopefully, these studies will make treatment of the disease by enzyme replacement feasible.

Receptor-mediated uptake of lysosomal enzymes may be part of the mechanism by which hydrolytic enzymes enter lysosomes. It has implications for enzyme replacement (i.e., the enzyme must have the proper structure to enter the target cells) and for the delivery of drugs to connective tissue cells. It opens up the possibility of disease in which hydrolytic enzymes are inappropriately located because the receptor is deficient, in addition to enzyme localization disorders such as mucolipidoses, in which the enzymes lack the recognition marker.

Proposed Course:

Studies of the chemical structure of the recognition marker by which lysosomal enzymes bind to the cell membrane will be continued. For technical reasons, the inhibitor of iduronidase uptake will be purified and analysed, rather than the enzyme itself.

The availability of an antiserum to α-L-iduronidase shall enable us to 1) simplify the purification of that enzyme by use of an antibody-sepharose column to supplement or replace some of the steps currently used; 2) identify cross-reactive proteins among patients with iduronidase deficiency diseases (i.e., the Hurler, Scheie and Hurler-Scheie syndromes) and perhaps to find some correlation between the degree of clinical severity and the presence of absence of CRM proteins; 3) follow the "life-cycle" of iduronidase by pulse-labeling cells with radioactive amino acids and sugars, and follow the path of labeled iduronidase from polysomes to lysosomes; 4) localize iduronidase by immunofluorescence, and verify the existence of a surface-bound pool of enzyme as well as of a lysosomal pool.

Kidney enzyme purified to homogeneity will be examined for subunit structure, amino acid and carbohydrate composition. An attempt will be made to convert the kidney enzyme (which is of the low uptake form) into a high-uptake form, by enzymatic modification, or by chemically coupling it to a protein fragment containing the recognition marker. Enzyme of the high uptake form (from urine) will be labeled with I^{125} in order to study binding to the cell receptor. An assay for the receptor will be developed and its purification undertaken.

Chromosomal mapping of arylsulfatase A will be extended to other sulfatase to resolve two questions: is there an autosomal locus in addition to the X-chromosomal locus of iduronate sulfatase? This question is raised by the finding of female Hunter patients. And is there a locus common to all sulfatases which would explain the genetic disease, multiple sulfatidosis, in which all sulfatases are deficient?

In collaboration with Dr. Irwin Leder (LBM) the measurement of heparan sulfatase activity will be improved by preparation and use of synthetic substrates. This enzyme is deficient in the Sanfilippo A syndrome, the diagnosis of which is difficult by presently available methods.

Publications:

1. Neufeld, E.F., Lim, T.W. and Shapiro, L.J.: Inherited Disorders of lysosomal metabolism, Ann. Rev. Biochem., 44: 357-376, 1975.

2. Shapiro, L.J., Hickman, S., Hall, C.W. and Neufeld, E.F.: Biochemical studies in Mucolipidoses II and III. March of Dimes-Original Articles Series 11 (6): 301-305, 1975.

3. Stevenson, R.E., Howell, R.R., McKusick, V.A., Suskind, R., Hanson, J.W., Elliott, D.E. and Neufeld, E.F.: The iduronidase deficient mucopolysaccharidoses; clinical and roentgenographic features. Pediatrics 57: 111-122, 1976.

4. Shapiro, L.J., Hall, C.W., Leder, I.G. and Neufeld, E.F.: The relationship of α-L-iduronidase and Hurler connective factor. Arch. Biochem. Biophys. 172: 156-161, 1976.

5. Neufeld, E.F., Liebaers, I. and Lim, T.W.: Iduronate sulfatase determination for the diagnosis of the Hunter syndrome and the detection of the carrier state. Adv. Exptl. Med. Biol. 68: 253-260, 1976.

6. Liebaers, I. and Neufeld, E.F.: Iduronate sulfatase activity in serum, lymphocytes and fibroblasts - simplified diagnosis of the Hunter syndrome. Pediat. Res. (In press).

7. McKusick, V.A., Neufeld, E.F. and Kelly, T.E.: The mucopolysaccharidoses storage diseases. In Stanbury, J.B., Wyngaarden, J.B. and Fredrickson, D.S. (Eds.): The Metabolic Basis of Inherited Disease. New York, McGraw-Hill, Fourth Edition, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41025-05 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies on the Cell Surface Receptor for IgE

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

PI:	H. Metzger	Chief, Section on Chem. Immun.	ARB NIAMDD
OTHER:	C. Isersky-Carter	Senior Staff Fellow	ARB NIAMDD
	G. Mendoza	Research Associate	ARB NIAMDD
	S. Newman	Staff Fellow	ARB NIAMDD
	G. Rossi	Visiting Scientist	ARB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Arthritis and Rheumatism

SECTION
Chemical Immunology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	5	1

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

We are pursuing an in-depth investigation on the structure and function of the membrane receptor for IgE. For these studies intact cells from a rat basophilic leukemia and a mouse mastocytoma as well as normal rat and mouse mast cells are being used. In addition receptor-bearing membrane particles and solubilized receptors are being studied, with the aim being to understand the structure of the receptor as well as its integration in the membrane. Results of such studies as well as direct functional studies are aimed at understanding the early steps in the cellular triggering by an antigen-induced antibody-mediated reaction.

PHS-6040
(12-75)

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. In addition we wish to understand the mechanism by which the ternary complex of antigen-IgE antibody--cell surface receptor triggers the degranulation of basophilic cells.

Methods Employed:

Cells:

In addition to the rat basophilic leukemia line which we have had in culture and, as solid tumors in young rats, a mouse mastocytoma has been successfully cultured. These cells as well as normal rat and mouse mast cells have been used for our studies.

Other:

The principal new method developed has been a simple assay for assessing the activity of the solubilized receptors (see below).

Major Findings:

Since our previous report the following has been accomplished:

1. Studies on intact cells.

   a. A detailed analysis of the IgE binding properties of mouse and rat, tumor and normal mast cells revealed

      i. that the numbers of receptors per cell were roughly equivalent

      ii. that the forward rate constants for all cells were roughly equivalent ($k_1 \tilde{=} 10^5$)

      iii. that the reverse rate constant for normal mouse cells was markedly different than the constant for the mouse mastocytoma and normal and tumor rat cells and that this is a property of the isolated receptor

iv. that none of the cells could distinguish between rat and mouse IgE.

b. A search for "cryptic" receptors revealed that all of a cell's receptors for IgE are expressed on the surface membrane, i.e. there are no active receptors elsewhere in the cell. This was studied by comparing the number of receptors on intact cells and detergent-solubilized cells (below).

2. Studies on membrane fragments.

a. Stability studies showed that the receptor in situ was stable at $4.4 \leq ph \leq 9.5$ but outside this range rapidly underwent irreversible changes; was stable at $\leq 45°C$ but agin was irreversibly altered above this temperature; was insensitive to a variety proteolytic enzymes and neuramidinase but was inactivated with phospholipase C (the latter may have resulted from an indirect effect).

b. Improved yields and increased purity was achieved in the preparations of membranes. Electron-microscopic examination showed the preparations to be excellent. Analysis of the binding properties suggests that all of the vesicles are "right-side-out" so that studies attempting to assess whether the receptor penetrates the membrane cells can not yet be performed.

3. Studies on solubilized receptors.

a. An assay was developed with which the presence of solubilized receptor can be detected based on the receptor's capacity to bind IgE. The assay takes advantage of the fact that free IgE is largely soluble in 40% saturated $(NH_4)_2SO_4$ but IgE bound to receptor (or antibody) is not.

b. The assay is very sensitive to changes in receptor concentration highly reproducible and relatively rapid. Using this assay:

i. conditions for solubilizing the receptor with non-ionic detergents could be optimized

ii. the stability of the receptor to a variety of conditions and reagents was assessed

iii. binding kinetics of receptor were analyzed

iv. molecular weight studies have been begun

v. methods for affinity-purifying the receptor are being assessed.

4. Antibody to receptor for IgE.

Serum of rabbits immunized with membrane fractions of the rat basophilic leukemia cells a) inhibited IgE binding by rat and mouse cells, and b) precipitates with solubilized saturated or un-saturated receptor.

Providing the antiserum can be made more specific it can be used as a probe of the integration of the receptor in the membrane and potentially for studying the role of the receptor in triggering of mast cells.

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 understand more completely 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. We now have what we believe is the first instance of a system in which a receptor for an immunoglobulin can be studied rigorously. With it we can potentially make significant contributions to our understanding of antigen-induced, antibody-mediated, cellular reactions.

Proposed Course:

1. Purification of receptor.
2. Further characterization of receptor: mol wt., amino acid composition, carbohydrate composition.
3. Further characterization of receptor integration in membrane.
4. Attempt to visualize solubilized receptor by electron-microscopy; similarly to visualize the receptor *in situ* by electron-microscopy.
5. Assess possible function of receptor as a Ca^{++} gating protein.
6. Begin study on role of Fc region in IgE binding.

Publications:

1. R.P. Siraganian, A. Kulczycki, Jr., G. Mendoza and H. Metzger. Ionophore A-23187 induced histamine release from rat mast cells and rat basophilic leukemia (RBL-1) cells. J. Immunol., 115 1599-1602, 1975.

2. Metzger, H., Budman, D., Lucky, P.: Interaction of IgE with rat basophilic leukemia cells V. Binding properties of cell free particles. Immunochem. (In press, 1976).

3. Metzger, H. and Bach, M.K.: The receptor for IgE on mast cells and basophils: Studies on IgE binding and on the structure of the receptor in Modern Concepts and Developments in Immediate Hypersensitivity. (M.K. Bach ed.) Marcel Dekker (In press, 1976).

4. Metzger H.: Receptors for IgE: Possible Transducers for Antibody Mediated Cell Activation in Cell Membrane Receptors for Viruses, Antigens and Antibodies, Polypeptide Hormones and Small Molecules, (R.F. Beers, Jr. and E.G. Bassett, eds.) Raven Press New York 1976 pp. 289-301.

5. Halper, J. and Metzger, H.: The Interaction of IgE with Rat Basophilic Leukemia Cells. VI Inhibition by IgGa Immune Complexes. 1976 (In Press).

6. DeLisi, C. and Metzger, H.: Some Physical Chemical Aspects of Receptor-Ligand Interactions. Immunol. Communications 1976 (In Press).

7. Mendoza, G.R. and Metzger, H.: Disparity of IgE Binding Between Normal and Tumor Mouse Mast Cells. J. Immunol. 1976 (In Press).

2. Metzger, H., Budman, D. and Lucky, P.: Interaction of IgE with rat basophilic leukemia cells V. Binding properties of cell free particles. Immunochem. (In press) 1976.

3. Metzger, H. and Bach, M.K.: The receptor for IgE on mast cells and basophils: Studies on IgE binding and on the structure of the receptor. In Bach, M.K. (Ed.): Modern Concepts and Developments in Immediate Hypersensitivity. New York, Marcel Dekker, (In press, 1976).

4. Metzger, H.: Receptors for IgE: Possible transducers for antibody mediated cell activation. In Beers Jr., R.F. and Bassett, E.G. (Eds.) Cell Membrane Receptors for Viruses, Antigens and Antibodies, Polypeptide Hormones and Small Molecules. New York, Raven Press, 1976, pp. 289-301.

5. Halper, J. and Metzger, H.: The interaction of IgE with rat basophilic leukemia cells. VI. Inhibition by IgGa immune complexes. Immunochemistry, (In press, 1976).

6. DeLisi, C. and Metzger, H.: Some physical chemical aspects of receptor-ligand interactions. Immunol. Communications. (In press, 1976).

7. Mendoza, G.R. and Metzger, H.: Disparity of IgE binding between normal and tumor mouse mast cells. J. Immunol. (In press, 1976).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41028-01 ARB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Renal effects of aspirin and other non-steroidal anti-inflammatory drugs in systemic lupus erythematosus

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

PI:	P. H. Plotz	Senior Investigator	ARB	NIAMDD
OTHER:	R. Kimberly	Clinical Associate	ARB	NIAMDD
	J. R. Gill, Jr.	Chief, Hypertension & Endo. Br.	IR EB	NHLI

COOPERATING UNITS (if any)
Hypertension and Endocrinology Branch, NHLI

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	3/4	1/4

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

Aspirin, an important drug in the therapy of many connective tissue diseases including systemic lupus erythematosus, has been found (Seaman, W. E. and Plotz, P. H., Arthr Rheum 19:155-160, 1976) to raise serum creatinine and BUN and lower creatinine clearance when administered to some patients with SLE as well as to some normal volunteers. Although this effect appears to be rapidly and completely reversible, if it is unrecognized it may falsely suggest deteriorating renal function due to activity of the underlying disease. Studies are underway to determine whether this effect is related to renal blood flow and tubular function, and whether it correlates with renal output of prostaglandin, since aspirin inhibits prostaglandin synthesis.

PHS-6040
(12-75)

Project Description:

Objectives:

To investigate the mechanism of the apparent alteration in renal function induced by aspirin.

Methods Employed:

In patients requiring aspirin therapy, careful studies of renal function are performed. In selected patients with systemic lupus erythematosus, studies include inulin and PAH clearances to determine GFR, RBF, and tubular function.

Major Findings:

Aspirin in therapeutic doses produces an acute fall in creatinine clearance in some patients with systemic lupus and in some normals.

Significance to Bio-Medical Research and the Program of the Institute:

Recognition of this effect of aspirin should prevent misinterpretation of fluctuation in renal function due to salicylates.

Proposed Course:

Studies to continue

Publications:

Seaman, W. E. and Plotz, P. H.: Effect of aspirin on liver tests in patients with RA or SLE and in normal volunteers. Arthritis Rheum 19: 155-160, 1976.

METABOLIC DISEASES BRANCH

The general goals of the Branch are to investigate the structure, secretion and biochemical mechanism of action of hormones governing ion transport and mineral metabolism and to apply the knowledge gained to understanding and treating related clinical disorders. Current investigations include hormone-receptor interaction, mechanism of receptor control of adenylate cyclase, cyclic AMP control of ion transport, and regulation of parathyroid hormone secretion. Systems under study include β-adrenergic receptors in several tissues and isolated parathyroid cells. Clinical studies on hyperparathyroidism have utilized extensively research findings from the laboratory. Radioimmunoassays for parathyroid hormone have been applied to determine the hormone in samples obtained selectively by venous catheterization, thereby allowing mobilization of parathyroid adenomas. Further studies continue on utilizing urinary cyclic AMP clearance as a clinical parameter of parathyroid hormone action. Still other studies under development will take advantage of an autologous parathyroid transplant program developed in collaboration with the Surgery Branch, NCI. Autologous transplants to the arm will allow direct sampling of draining veins for parathyroid hormone secretion in man under conditions wherein the tissue can be selectively challenged with potential secretogogues or inhibitors of parathyroid secretion.

β-adrenergic Receptor of the Avian Erythrocytes.

The interaction of catecholamines with the β-adrenergic receptor and activation of adenylate cyclase were studied with the plasma membrane of turkey erythrocytes. Interaction of β-adrenergic agonists with receptor in these cells causes specific stimulation of sodium and potassium transport. This effect is mediated through the adenylate cyclase-cyclic AMP system and can be reproduced by adding exogenous cyclic AMP. The effect of catecholamines on adenylate cyclase, cyclic AMP accumulation and sodium or potassium transport is specific for the levorotatory form of these compounds. Activation in the system by catecholamines is specifically inhibited by (-)stereoisomers of β-adrenergic blockers.

The β-adrenergic receptor site has been identified using a radioiodinated analog of a β-blocker designed for this purpose. Hydroxybenzylpindolol (1-(1-para-hydroxyphenyl-2-methyl-2-propylamino)-3-(4-indolyloxy)-2-propanol) is a high-affinity β-blocker containing a phenolic group. The latter compound can be radioiodinated to high specific activity and binds specifically to a site on turkey erythrocyte membranes. The structure of the iodinated product was proven by mass spectroscopy to be monoiodohydroxybenzylpindolol with the iodine on the phenolic group. Kinetic and equilibrium studies identified a single class of high affinity ($2\text{-}4 \times 10^{10} M^{-1}$) receptor sites on turkey erythrocyte membranes, 200-400 sites per cell. There was no evidence for negative cooperativity. A series of β-adrenergic agonists and inhibitors compete for this binding site with affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. Receptor occupancy for agonists correlated

linearly with function (cyclic AMP production). The stereospecificity of interaction with the iodinated β-blocking agent and the correspondence between affinity for site and biological effectiveness indicate that binding of iodohydroxybenzylpindolol validly reflects interaction with the β-adrenergic receptor.

Calcitonin receptors were identified on cultured human lymphocytes. The characteristics of these receptors are similar to those on rat renal and calvarial membranes as determined by competitive binding studies. The lymphocyte receptors, however, do not appear capable of modulating adenylate cyclase enzyme in that cyclic AMP was not detected after adding calcitonin to intact cells. Lymphocyte preparations should prove useful, however, in that they represent a readily obtainable human tissue containing calcitonin receptors. Results with this human indicated that, although calcitonin polypeptides have undergone remarkable evolutionary changes in structure and effectiveness, there has been no significant corresponding change in biological nature of calcitonin receptors.
[Drs. Marx and Aurbach, NIAMDD; Dr. Marc Lippman, MO, NCI]

Purine nucleotides enhance catecholamine-stimulated adenylate cyclase activity in the erythrocyte membrane. A guanine nucleotide site separate from the catalytic site for ATP regulates adenylate cyclase activity. Although guanine nucleotides (the most effective is guanylylimidodiphosphate, Gpp(NH)p) enhance isoproterenol-activated adenylate cyclase with apparent increase in sensitivity to the agonist, there is nevertheless no change in affinity of the receptor.
[Drs. Spiegel, Brown and Aurbach, NIAMDD: Dr. David Rodbard, NICHD: Dr. Daniel Hauser, Sandoz, Basle]

Prior incubation of adenylate cyclase with Gpp(NH)p leads to a highly and persistently activated "holocatalytic" state. This activated state, however, shows no change in affinity of ligands for receptor. It is concluded that guanine nucleotides act on adenylate cyclase in the turkey erythrocyte system at a site distal to the receptor. Recent studies indicate that the enzyme reaction itself may involve vicinal dithiol mechanism. Arsenite was found to be an effective inhibitor of the adenylate cyclase reaction and this inhibition was specifically potentiated by dimercaptopropanol (BAL). [Dr. Spiegel]

Clinical studies are continuing on primary hyperparathyroidism and its familial variants. Detailed family screening and case finding has produced 12 kindreds for analysis. These studies have allowed segregation of the familial variants into two apparently distinct disease syndromes -familial multiple endocrine adenomatosis (MEN I) and familial hyperparathyroidism (FH). FH was distinguished from MEN I by 1) a higher incidence of hypercalcemia below the age of 20 and virtually a 100% penetrance for hypercalcemia after age 20, 2) milder clinical manifestations - none had recurrent nephrolithiasis or recurrent peptic ulceration, 3) negligible hypercalciuria, 4) normal basal concentrations of glucagon and gastrin, and 5) poor response to subtotal parathyroidectomy. Distinction between the two syndromes, both

apparently inherited as autosomal dominant traits, is important because in FH the clinical course is generally milder and subtotal parathyroidectomy is less likely to be beneficial.
[Drs. Marx, Spiegel, Brown and Aurbach]

Studies on modes of localizing parathyroid tumors continue. A high degree of success has been obtained in localizing tumors through vascular catheterization procedures. Parathyroid arteriography developed and performed by Dr. John Doppman afforded in approximately 45% of cases in which it was used, the identification of abnormal masses of tissue subsequently identified as parathyroid. Confirmation of the mass thus identified as parathyroid tissue can be accomplished by verifying high concentrations of parathyroid hormone by radioimmunoassay in veins draining the lesion. Draining vessels contain concentrations of hormone 2- to 20-fold or more greater than the concentration of parathyroid hormone detected by immunoassay in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in the one vein draining the lesion. Contralateral veins show conconcentrations equal to samples obtained from the general circulation. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on either side greater than the concentrations of peripheral plasma. 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, Spiegel, Brown, and Aurbach, NIAMDD: Dr. Doppman, Radiology Dept., C.C.]

A method has been developed for isolating functionally intact bovine parathyroid cells. The cells are obtained after 30-60 minutes of digestion of tissue with a mixture of collagenase and DNAse with periodic mechanical destruction by vigorous pipetting. The cell population produced is indistinguishable from that of intact bovine tissue by electron microscopy and shows a similar inverse proportionate control of hormone secretion in response to calcium. Studies with the isolated parathyroid cells proved that they contain beta-adrenergic receptors (identified by specific binding studies with iodohydroxybenzylpindolol) and respond to beta-adrenergic catecholamines with a 300% increase in parathyroid hormone secretion and sharp increase in cyclic AMP concentration. This study affords proof that the parathyroid cell itself contains catecholamine-regulated adenylate cyclase activity (prior studies using parathyroid slice preparations could not exclude the possibility that cyclic AMP response was a function of contaminating fat cells).
[Drs. Brown and Hurwitz]

Osteoporosis Studies

Clinical studies of osteoporosis have been continued, using photon absorption densitometry, with observations of the bone mineral content in various populations and of the possible effect thereon of dietary manipulation. In a normal population measurements in many individuals of various ages indicated linear increase in bone mineral content into the third decade, thereafter a

decrease which is more rapid in women than in men. Blacks were noted to have a greater bone mineral content than whites and to lose mineral at a slower rate, in confirmation of the observations of others that osteoporosis is uncommon in normal blacks. Observations in black children with sickle cell anemia revealed a high proportion of decreased bone mineral content, particularly in males. In ten white patients with osteoporosis, a high calcium, high phosphorus diet for one year produced no change in bone mineral content; it was uncertain whether this result meant ineffectiveness of the high mineral intake or insensitivity of the densitometric method to small changes in mineral content. [Dr. G. D. Whedon et al.]

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 43,001-11 MD

PERIOD COVERED
July 1, 1976 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies in Bone Metabolism

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

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
A. M. Spiegel, M.D., Clinical Associate, MDB, NIAMDD
J. M. Phang, M.D., Metabolism Branch, NCI
G. D. Whedon, M.D., Director, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
E. M. Brown, M.D., Clinical Associate, MDB, NIAMDD
J. R. Shapiro, M.D., Chief, Section on Endocrinology, Greater Southeast Community Hospital, Washington, D. C.
W. T. Moore, M.D., Washington Hospital Center, Washington, D. C.

COOPERATING UNITS (if any)
Dr. Mones Berman, Mathematical Research Branch, NIAMDD
Metabolism Branch, NCI
Greater Southeast Community Hospital, Washington, D. C.

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.75	.25	.50

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

The objectives of the project on bone metabolism are to investigate the factors influencing mineral storage and loss in demineralizing bone diseases, to investigate the response of the parathyroid gland to rapid changes in blood calcium, and to study the influence of added calcium and phosphate on resorption of bone mineral. The project involves metabolic balance studies. It also involves 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, and estimation of bone density by photon absorptiometry.

During the past year, clinical studies of osteoporosis have been continued, using photon absorption densitometry, with observations of the bone mineral content in various populations and of the possible effect thereon of dietary manipulation.

PHS-6040

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. (3) Estimation of bone density in the radius by photon absorptiometry.

Major Findings: Studies have continued in order to evaluate the clinical usefulness of photon absorption method of bone mineral analysis. Using this technique, with an iodine-125 source, values for bone mineral content in a normal population of children and adults were determined. These studies demonstrated a fairly linear increase in bone mineral content from birth into the third decade; thereafter bone mineral decreased in both men and women, but at a faster rate in women than in men.

The effect on bone mineral content of a high calcium-high phosphorus intake (diet supplemented to total of 2400 mg. calcium, 2200 mg. phosphorus) was evaluated in a group of ten osteoporotic females. This medication was continued for one year and was then supplemented with vitamin D. The high calcium-high phosphorus intake produced no significant change in bone mineral content as measured in the distal radius by the photon absorption technique. At this time it cannot be determined whether this study of high mineral intake suggests that the photon absorption method is insensitive with regard to small changes in mineral content or that the therapeutic regimen as described was ineffective.

Measurement of bone mineral content in the radius in black populations from birth through adult life disclosed a greater mineral content in blacks compared with whites at all ages. Based on group data (single or few measurements each on many individuals), black females lose mineral in the radius with aging. These studies confirm the clinical impression that osteoporosis is very uncommon in blacks and suggests that as currently defined osteoporosis should not occur as a primary disease in black males. Bone mineral content was determined in black patients with sickle cell anemia. Sixty-four percent of males and 39 percent of females with sickle cell disease have less bone mineral than normal black children of the same age. The ratio of bone mineral content to bone width followed the normal pattern in these children. Loss of bone mineral content did not correlate with severity of anemia or transfusion requirement.

Publications:

1. Moore, W. T., Shapiro, J. R., Jorgensen, H., Reid, J., Epps, C. H., and Whedon, G. D.: The evaluation of bone density findings in normal populations and osteoporosis. Trans. Am. Clin. Climatol. Assoc. 87: 128-138, 1975.

2. Epps, C. H., Jr., Shapiro, J. R., Moore, W. T., and Jorgensen, H.: In vivo bone mineral content in sickle cell anemia. Proceedings of the First National Symposium on Sickle Cell Disease. Washington, D. C., June 27-29, 1974, National Institutes of Health, pp. 307-309, DHEW Publication No. (NIH) 75-723.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 43,002-11 MD

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Study of parathyroid hormone: Physiological regulation of secretion; physical, chemical, and immunochemical properties; biochemistry; and structure, and mechanism of action.

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

G. D. Aurbach, M.D., Chief, MDB, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
A. M. Spiegel, M.D., Clinical Associate, MDB, IAMDD
E. M. Brown, M.D., " "
J. O. Koehler, M.D. " "
J. T. Potts, Jr., M.D., Massachusetts General Hospital, Boston.
H. T. Keutmann, M.D. "
H. Niall, M.D. "

COOPERATING UNITS (if any)

Endocrine Unit, Massachusetts General Hospital.

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.25	1.75	1.50

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

Little is known about the nature of circulating parathyroid hormone in clinical disease. It is the purpose of this project to isolate parathyroid hormone, determine the structure of the polypeptide, elucidate the mechanism of action of the hormone, and to develop a clinically useful test for circulating parathyroid hormone. From these studies it is expected that one can understand the pathophysiology of certain metabolic diseases of bone and endocrine disturbances. The entire structures of bovine and porcine parathyroid hormone have been determined as well as the first 37 residues of human parathyroid hormone. Synthetic polypeptides representing the first 34 residues of bovine and human parathyroid hormone have been prepared. These molecules show all the biological properties of the native hormonal polypeptide. The highly sensitive specific radioimmunoassay for the hormone has been developed and is being modified further for potential use as a highly specific clinical tool. Recent studies show that the mechanism of action of purified hormone is mediated through direct hormonal activation of adenylate cyclase in bone and kidney. An isolated parathyroid cell system has been developed that allows studies of secretogogue for parathyroid hormone.

PHS-6040
(12-75)

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 by methods previously described from this laboratory. The hormone is assayed in vivo in rats, in vitro by measuring hormonal activation of adenylate cyclase, or by a highly sensitive radioimmunoassay. Adenylate cyclase is measured by determining conversion of α-^{32}P-labeled ATP to cyclic 3',5'-AMP. Endogenous concentration of cyclic 3',5'-AMP or cyclic 3',5'-GMP is determined by radioimmunoassay.

Major findings: Mechanism of Action. Our earlier studies showed 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 adenylate cyclase in the kidney as well as in bone, the two organs known to be physiological receptors for action of the hormone. Adenylate cyclase in the kidney is found in two different anatomic zones; adenylate cyclase in the cortex responds specifically to parathyroid hormone whereas that in the medulla responds to vasopressin. Further studies show that virtually all the adenylate 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 from indirect in vivo physiological experiments.

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 indicated approximately eight amino acid substitutions in the human hormone. The amino acid sequence has been completed for the first 37 amino acids at the amino-terminal end of the molecule (Dr. Hugh Niall, MGH, Boston). Based on this analysis, Dr. Geoffrey Tregear (MGH, Boston) synthesized the tetratriacontapeptide representing the amino terminus of the molecule. The product is fully as active in vitro as in vivo as the hormone isolated from human parathyroid adenomas.
[G.D.Aurbach; Drs.Potts,Niall,O'Riordan and Keutmann]

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 a further 1 to 2 mg of human parathyroid hormone can be isolated in pure form it will be possible to determine the amino acud sequence in the C-terminal region represented by the remainder of the molecule, residues 38-84.

Parathyroid cells in vitro: Methods have been developed for isolating functionally intact bovine parathyroid cells. Finely minced tissue is digested for 30-60 minutes with collagenase and DNase and periodically disrupted by vigorous pipetting. The resulting cell population is indistinguishable from intact bovine tissue by electron microscopy and shows a similar inversely proportionate relationship of parathyroid hormone (PTH) release to calcium.

Isolated bovine parathyroid cells also have an intact β-adrenergic receptor as shown by up to a 300% increase PTH release by 10^{-6} M (-)isoproterenol. The order of potency isoproterenol > epinephrine >> norepinephrine suggests β_2-adrenergic receptor characteristics. A similar order of potency of agonists is seen in cAMP production either in intact cells or in membranes prepared from isolated parathyroid cells. Binding studies employing the iodinated β-adrenergic blocker ^{125}I-hydroxybenzylpindolol (I-HYP) facilitate direct identification and characterization of bovine parathyroid β-adrenergic receptors. At equilibrium ^{125}I-HYP binds to 5000-10,000 sites per cell with affinity constant (K) of $3\text{-}4\times10^{10}\ M^{-1}$. Furthermore, potency in inhibition of ^{125}I-HYP binding to membranes or intact cells is very similar to potency of various ligands as activators or inhibitors of both adenylate cyclase and PTH release. Hence there appears to be a one-to-one relationship between hormone binding, adenylate cyclase activation and release of PTH.

Initial studies employing isolated cells from pathologic human parathyroid glands removed at the time of surgery for hyperparathyroidism suggest a variety of abnormalities in function. Cells from patients with both adenomas and primary hyperplasia suppress to 20-40% of their maximal hormone output, but adenomas appear to require a higher than normal calcium to suppress. The cells from several patients have failed to respond to isoproterenol with PTH release although they do respond with about 10% of the cAMP response of normal bovine tissue.

These studies indicate the utility of isolated parathyroid cells for investigating the physiology of both the normal and abnormal parathyroid and proves that the parathyroid cell itself contains catecholamine-regulated adenylate cyclase activity (prior studies using parathyroid slice preparations could not exclude the possibility that cyclic AMP response was a function of contaminating fat cells).
[Drs. Brown and Hurwitz]

Significance to Biomedical Research and Program of the Institute: Same as for July 1968-June 1975.

Proposed Course of Project: Same as for July 1968-June 1975.

Publications:

1. Aurbach, G.D., and Chase, L.R.: Cyclic nucleotides and biochemical actions of parathyroid hormone and calcitonin. In Parathyroid Gland, edited by G.D.Aurbach. Washington, D.C., American Physiological Society, 1976, pp. 353-381. (Handbook of Physiology, Endocrinology Section (R.O.Greep and E.B.Astwood, Editors), Section 7, Vol. VII.

2. Aurbach, G.D.: Introduction. In Parathyroid Gland, edited by G.D. Aurbach. Washington, D. C., American Physiological Society, 1976, pp. 1-2. (Handbook of Physiology, Endocrinology Section (R.O.Greep and E.B.Astwood, Editors), Section 7, Vol. VII.

3. Aurbach, G.D.: Hormone receptors in the kidney. In Bolis, L., Hoffman, J., and Leaf, A. (Eds.): Membranes and Diseases. New York, Raven Press, 1976, in press.

4. Desbuquois, B., and Aurbach, G.D.: Use of polyethylene glycol to separate free and antibody-bound peptide hormones in radioimmunoassays. In Antoniades, H.H. (Ed.): Hormone in Human Plasma. Cambridge, Massachusetts, Harvard University Press, 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 43,003-11 MD

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies on the Mode of Action of Thyrocalcitonin

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

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Stephen J. Marx, M.D., Senior Investigator, MDB, NIAMDD
Edward M. Brown, M.D., Clinical Associate, MDB, NIAMDD
Marc Lippman, M.D., MO, NCI

COOPLRATING UNITS (If any)

Medical Oncology Branch, NCI

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	.50	1.00

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

The purpose is to study the interaction of calcitonin with its specific receptor target organs. 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 for characterizing hormone receptors in kidney, bone and other tissues. Studies are in progress to characterize further the interaction of calcitonin with tissue receptors. It also will be of interest to solubilize the receptors and characterize them chemically.

PHS-6040
(12-75)

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 tissue and to lymphoid cells isolated from human and other species or grown in tissue culture.

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 binding to 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. The interaction of the hormone-receptor complex with the adenylate cyclase enzyme is modified by the presence of guanine nucleotides. In the presence of GMPPNP the threshold for hormonal activation is lowered.

The relative renal responses to calcitonin analogs are similar whether determined by competitive binding or adenylate cyclase activation. Thus potency differences of the analogs tested are largely dependent upon effectiveness at the receptor level. Activity of the adenylate cyclase enzyme of human lymphocytes was not modulated by ambient or bound calcitonin.

Calcitonin receptors were identified on cultured human lymphocytes. The characteristics of these receptors are similar to those on rat renal and calvarial membranes as determined by competitive binding studies. The lymphocyte receptors, however, do not appear capable of modulating adenylate cyclase enzyme in that cyclic AMP was not detected after adding calcitonin to intact cells. Lymphocyte preparations should prove useful, however, in that they represent a readily obtainable human tissue containing calcitonin receptors. Results with this human indicated that, although calcitonin polypeptides have undergone remarkable evolutionary changes in structure and effectiveness, there has been no significant corresponding change in biological nature of calcitonin receptors.

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, bone, and other tissues.

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. Purified lymphoid cell types are being characterized with respect to the density of calcitonin receptors on their surface. The whole cell system also may be applied to evaluate the regulation of receptor metabolism in vitro and in human disease states.

Publications:

1. Marx, S.J., and Aurbach, G.D.: Renal receptors for calcitonin: coordinate occurrence with calcitonin-activated adenylate cyclase. Endocrinology 97: 448-453, 1975.

2. Potts, J.T., Jr., and Aurbach, G.D.: Chemistry of the calcitonins. In Parathyroid Gland, edited by G.D.Aurbach. Washington, D.C., American Physiological Society, 1976, pp. 423-430. (Handbook of Physiology, Endocrinology Section (R.O.Greep and E.B.Astwood, Editors), Section 7, Vol. VII.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 43,004-11 MD

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies on pseudohypoparathyroidism and related disorders

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

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
A. M. Spiegel, M.D., Clinical Associate, MDB, NIAMDD
E. M. Brown, M.D., " "
J. O. Koehler, M.D. " "

COOPERATING UNITS (if any)

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.50	.25	.25

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

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. Our findings that parathyroid hormone action is likely mediated through activation of adenylate cyclase, led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3',5'-AMP in the urine. In normal subjects as well as usual forms of hypoparathyroidism, parathyroid hormone causes a 10- to 60-fold increase in urinary cyclic AMP (cAMP). This response is abnormal (slight or no increased cAMP excretion) in pseudohypoparathyroidism, indicating that there is a defective parathyroid hormone-adenylate cyclase complex in the kidney in this disorder.

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 adenylate 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. Radioimmunoassay 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: In normal subjects, parathyroid hormone causes a 10- 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 pseudopseudohypoparathyroid 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 rate 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 transmission of the disorder. It appears likely that the cause of the disorder may be the existence of genetically deficient or defective adenylate 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-adenylate 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 July 1, 1968-June 30, 1975.

Proposed Course of Project: Same as reported for July 1, 1968-June 30,1975.

Publications:

1. Spiegel, A.M., Di Chiro, G., Gorden, P., Ommaya, A.K., Kolins, J., and Pomeroy, T.C.: Diagnosis of radiosensitive hypothalamic tumors without craniotomy. Ann. Intern. Med., 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 43,005-11 MD

PERIOD COVERED
July 1 , 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Study of Hormone-Mediated Solute Transport: Interaction of Hormones with Cell Receptors: Interrelationship between Biogeneration of Cyclic AMP and Active or Facilitated Transport.

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

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COOPERATING UNITS (if any)

Section on Gastroenteriology, Digestive Diseases Branch, NIAMDD

LAB/BRANCH
Metabolic Diseases Branch
SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.25	1.75	1.50

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

To isolate and purify specific hormone receptors in particular receptor tissues; to map function of the receptor against activation of adenylate cyclase; to determine the nature of the link between generation of cyclic 3',5'-AMP and solute transport in particular cell systems. β-adrenergic receptors have been identified using a radioiodinated analog of a β-blocker designed for this purpose. Hydroxybenzylpindolol (1-(1-para-hydroxyphenyl-2-methyl-propylamino)-3-(4-indolyloxy)-2-propanol) is a high-affinity β-blocker containing a phenolic group. The latter compound can be radioiodinated to high specific activity and binds specifically to β-receptor sites on cell membranes. Kinetic and equilibrium studies identified a single class of high affinity ($2-4 \times 10^{10} M^{-1}$) receptor sites on turkey erythrocyte membranes, 200-400 sites per cell. A series of β-adrenergic agonists and inhibitors compete for this binding site with affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. Receptor occupancy for agonists correlated linearly with function (cyclic AMP production). The stereospecificity of interaction with the iodinated β-blocking agent and the correspondence between affinity for site and biological effectiveness indicate that binding of Iodo-hydroxybenzylpindolol validly reflects interaction with the β-adrenergic receptor.

Objectives: To isolate and purify specific hormone receptors in particular receptor tissues; to map function of the receptor against activation of adenylate cyclase; to determine the nature of the 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. Results are analyzed by double reciprocal, Scatchard, or four-parameter logistic (Rodbard) models utilizing computer programs based on these models. 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.

The interaction of catecholamines with the β-adrenergic receptor and activation of adenylate cyclase were studied with the plasma membrane of turkey erythrocytes and mammalian reticulocytes. Interaction of β-adrenergic agonists with receptor in turkey erythrocytes causes specific stimulation of sodium transport. This effect is mediated through the adenylate cyclase-cyclic 3',5'-AMP (cAMP) system and can be reproduced by adding exogenous cAMP. Catecholamine-enhanced sodium transport is specifically inhibited by β-adrenergic blockers.

Incubation of turkey erythrocyte membranes with the GTP analog, GMPPNP, and isoproterenol leads to persistent activation of adenylate cyclase ("holocatalytic state") even after repeated sedimentation and resuspension of membranes in buffer without agonist. Conditions that prevent GMPPNP binding (reduced temperature, GTP, 1 mM EDTA) prevent formation of the holocatalytic state. Beta-adrenergic blockers can prevent formation of this state but are ineffective in reversing it once it has been formed.

The affinity and number of β-adrenergic receptors as determined with I-HYP are identical in the holocatalytic state as compared with control membranes. This indicates that formation of the holocatalytic state with GMPPNP involves a modification in the adenylate cyclase complex distal to the hormone receptor.

Adenylate cyclase activity is sensitive to monothiol inhibitors such as organic mercurials and N-ethyl maleimide. Vicinal dithiol inhibitors, arsenite and cadmium were found to inhibit adenylate cyclase activity in turkey erythrocyte and rat reticulocyte membranes. Cadmium proved to be a potent inhibitor ($K_i \sim 10^{-4}$ M). Inhibition by cadmium could be prevented by chelators such as EGTA or EDTA. Arsenite alone was a weak inhibitor (7 mM → only 70% inhibition) but its effect was greatly potentiated by equimolar 2,3 dimercaprol (BAL). Monothiols such as mercaptoethanol were much less effective in potentiating arsenite inhibition. Excess BAL could prevent or reverse both arsenite and cadmium inhibition and

was more effective than mercaptoethanol. The inhibitors were effective against basal, catecholamine, and fluoride-stimulated membrane-bound enzyme as well as against a detergent solubilized enzyme preparation, but had no significant effect on binding to the β-adrenergic receptor. These results indicate that vicinal dithiol groups are involved in the catalytic function of adenylate cyclase.

β-adrenergic receptor sites have been identified using a radioiodinated analog of a β-blocker designed for this purpose. Hydroxybenzylpindolol (1-(1-para-hydroxyphenyl-2-methyl-2-propylamino)-3-(4-indolyloxy)-2-propanol) is a high affinity β-blocker containing a phenolic group. The latter compound can be radioiodinated to high specific activity and binds specifically to a site on turkey erythrocyte membranes. A series of β-adrenergic agonists and inhibitors compete for this binding site with apparent affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. The stereospecificity of interaction with the iodinated β-blocking agent and the correspondence between affinity for site and biological effectiveness reflect validly interactions with β-adrenergic receptors.

β-Adrenergic Receptor of Mammalian Erythrocytes

The mature nonnucleated mammalian erythrocyte lacks adenylate cyclase activity. Immature rat erythrocytes (reticulocytes), however, possess significant adenylate cyclase activity which can be stimulated by catecholamines, GMPPNP and sodium fluoride. The reticulocytes contain β-adrenergic receptors identified with radioiodinated hydroxybenzylpindolol (I-HYP) as ligand. Antagonists and agonists display stereospecificity ((-)>>(+)) and orders of potency as inhibitors of I-HYP binding with rat reticulocyte membranes that parallel effects on adenylate cyclase activity. During red cell maturation, there is a rapid loss ($\sim$ 10% remaining after 1 week) of adenylate cyclase activity (including basal catecholamine- and fluoride-stimulated enzyme). β-adrenergic receptors are lost at a slower rate than adenylate cyclase activity during maturation. These studies provide evidence that hormone receptor and catalytic moiety are discrete entities.

Significance to Biomedical Research and Program of the Institute: 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 inter-connecting links between these cellular events are 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 interconnecting substance between binding and adenylate cyclase activation; isolation of the guanine nucleotide binding protein; identification of the system activated by cyclic AMP in stimulating transport of solute.

Publications:

1. Spiegel, A.M., Brown, E.M., Fedak, S.A., Woodard, C.J., and Aurbach, G.D.: Holocatalytic state of adenylate cyclase in turkey erythrocyte membranes: formation with guanylylimidodiphosphate plus isoproterenol without effect on affinity of β-receptor. J. Cyclic Nucleotide Res. 2: 47-56, 1976.

2. Brown, E.M., Aurbach, G.D., Hauser, D., and Troxler, F.: Beta-adrenergic receptor interactions: characterization of iodohydroxybenzylpindolol as a specific ligand. J. Biol. Chem. 251: 1232-1238, 1976.

3. Brown, E.M., Fedak, S.A., Woodard, C.J., Aurbach, G.D., and Rodbard, D.: Beta-adrenergic receptor interactions: direct comparison of receptor interaction and biological activity. J. Biol. Chem. 251: 1239-1246, 1976.

4. Aurbach, G.D., Brown, E.M., Spiegel, A.M., and Gardner, J.D.: Beta-adrenergic receptors, cyclic AMP and ion transport in the avian erythrocyte. In Beers, R.F., Jr., and Bassett, E.G. (Eds.): Cell Metabolism Receptors for Viruses, Antigens and Antibodies, Polypeptide Hormones, and Small Molecules. New York, Raven Press, 1976, in press.

5. Aurbach, G.D.: Hormone receptors, cyclic nucleotides and control of cell function. In Freinkel, N. (Ed.): The Year in Metabolism. New York, Plenum Press, in press.

6. Gardner, J.D., Aurbach, G.D., Spiegel, A.M., and Brown, E.M.: Receptor function and ion transport in turkey erythrocyte. Recent Progr. Hormone Res. 32: in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 43,006-01 MD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Study of hyperparathyroidism: Etiology, diagnosis and treatment.

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

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LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	1.00	.75

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

The project goal is the evaluation and treatment of hyperparathyroidism. Patients with persistent postoperative hyperparathyroidism are referred for evaluation and treatment. Hereditary hyperparathyroidism in particular is under investigation in the hopes of delineating hereditary molecular abnormalities in glandular regulation, as exemplified in the multiple endocrine neoplasia syndromes. Evaluation ranges from epidemiologic studies of families to in-house studies of patients and to in vitro analyses of excised tissue. Techniques currently being employed and improved include radioimmunoassay of parathyroid hormone, selective arteriography and selective venous sampling for parathyroid hormone, parathyroid gland cryopreservation and auto-transplantation, and transcatheter parathyroid gland infarction.

PHS-6040
(12-75)

Objectives: To study the regulation of parathyroid gland function. To develop and evaluate diagnostic tests of parathyroid function. To develop new methods for localization of hyperfunctioning parathyroid tissue. To evaluate methods of treatment of hyperparathyroidism.

Methods employed: Patients with suspected hyperparathyroidism are referred for evaluation and treatment. Evaluation includes radioimmunoassay of circulating parathyroid hormone, and of plasma and urinary cyclic AMP. Where appropriate, preoperative evaluation includes selective arteriography and venography with selective sampling for PTH determination. In selected cases adenomatous parathyroid glands are ablated by transarterial infusion of various agents. Parathyroid exploration is done by the Surgery Branch, NCI. If postoperative hypoparathyroidism is considered highly likely, parathyroid tissue may be simultaneously autotransplanted to a forearm muscle. Excised parathyroid tissue either fresh or cryopreserved is evaluated in vitro after preparation of dispersed cells. If postoperative hypoparathyroidism is considered possible, tissue is cryopreserved for subsequent autotransplantation. Techniques for cryopreserving and grafting parathyroid tissue are being studied in an experimental model with adult dogs.

Major findings: Diagnosis and clinical findings: A large number of patients with primary hyperparathyroidism have been treated and their clinical and chemical findings have been described. Weakness was noted more commonly than had previously been appreciated and muscle biopsy showed atrophy of type I and especially type II fibers in a manner diagnostic of denervating disease. Studies of neuromuscular function in subjects with secondary hyperparathyroidism showed similar clinical and histologic findings. The most useful diagnostic tests for hyperparathyroidism have been hypercalcemia associated with nonsuppressed or increased parathyroid hormone concentrations in peripheral blood and increased urinary excretion of cyclic AMP. These findings revert rapidly to normal following successful parathyroidectomy. Intestinal absorption of calcium is increased preoperatively and undoubtedly has a central role in causing hypercalcemia.
[G.D.Aurbach, S.J.Marx, A.M.Spiegel, E.M.Brown, and J.O.Koehler, MDB,NIAMDD; J.Sode, U.S.Naval Hospital; M.Brennan,Surgery Branch, NCI]

Familial hyperparathyroidism: Surveys of families of patients with primary parathyroid hyperplasia show that hereditary disease is very common (approximately 50% in our series of 25 index cases with primary parathyroid hyperplasia). In two large families a novel form of hyperparathyroidism was found wherein hypercalcemia occurs without hypercalciuria. Unlike typical cases of familial hyperparathyroidism, the penetrance of hypercalcemia approached 100% in the first two decades with severe expression even in one neonate. Review of results of surgery at the N.I.H. and elsewhere has shown that all of 6 patients in this category treated by subtotal parathyroidectomy had persistent postoperative hypercalcemia. While elevated concentrations of glucagon and/or gastrin were common in subjects with familial multiple endocrine neoplasia type I, these were not elevated in hypercalcemic members of these two families.
[S.J.Marx, A.M.Spiegel, E.M.Brown, J.O.Koehler, and G.D.Aurbach, MDB,NIAMDD; J.Gardner, DDB,NIAMDD; L.Recant, V.A.Hospital,Washington,D.C.]

Application of new radiologic techniques: 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 vein and thence through the major veins to reach the neck where selective samples are taken from each inferior 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 samples from the general circulation. 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 nonparathyroid cancers.

Thyroid and internal mammary arteriography enables preoperative identification of hyperfunctioning parathyroid tissue in 30% or more of cases and is also helpful in defining venous drainage to facilitate selective catheterization. In three cases of mediastinal parathyroid adenoma treatment was accomplished by transarterial infusion of hypertonic glucose, gelfoam or autologous clot to occlude the catheterized parathyroid artery. Two appear fully cured and one partially. There was no significant morbidity to these patients who would otherwise have required sternotomy.
[G.D.Aurbach, S.J.Marx, A.M.Spiegel, E.M.Brown, and J.O.Koehler, MDB,NIAMDD; J.Doppman, Radiology Dept., C.C., NIH]

Autotransplantation and cryopreservation of parathyroid: Persistent postoperative hyperparathyroidism or hypoparathyroidism are potential problems in patients with primary parathyroid hyperplasia or previous unsuccessful parathyroid exploration. These problems may be lessened if parathyroid tissue can be cryopreserved. We have developed experience with cryopreservation and autotransplantation of parathyroid glands in normal dogs and also in a few patients who developed permanent hypoparathyroidism postoperatively. Avoidance of tissue trauma or rapid changes in temperature or pH were essential for tissue preservation. Canine parathyroid tissue has been successfully autotransplanted after cryopreservation for as long as nine months and function has been documented by measuring release of immunoreactive PTH and by induction of hypocalcemia following graft excision.
[G.D.Aurbach, S.J.Marx, A.M.Spiegel, E.M.Brown, and J.O.Koehler, MDB,NIAMDD; G.Leight, H.Sears, and M. Brennan, Surgery Branch, NCI]

Significance to Biomedical Research and Program of the Institute: As an outgrowth of these studies, methods of localizing and treating hypersecreting tissues of several types are being improved. Hyperparathyroidism is a prototype of endocrine hyperfunction. Some patients have hyperparathyroidism in combination with hereditary hyperfunction of other endocrine glands including pituitary and pancreas. These hyperfunctions are usually benign but occasionally become cancerous. By studying families with hereditary disease we hope to gain insight into specific molecular lesions that cause loss of normal cellular regulation in these tissues. This collaborative program offers comprehensive treatment for a select group of patients who have proven difficult or impossible to treat in other centers in the United States and abroad.

Proposed Course of Project: Patients with suspected primary hyperparathyroidism will be accepted for evaluation and treatment. Continued efforts will be made to improve techniques for assessment of parathyroid hormone by radioimmunoassay or bioassay. We will continue to explore new methods of localizing hyperfunctioning parathyroid tissue. The applicability of parathyroid embolization and the role of parathyroid autotransplantation will be investigated. We will continue to study familial patterns of hyperparathyroidism. Patients in this program will provide a source of abnormal parathyroid glands to be studied in detail in vitro. Patients with parathyroid transplants will be ideal test subjects for putative parathyroid secretogogues.

Publications:

1. Spiegel, A.M., Marx, S.J., Doppman, J.L., Beazley, R.M., Ketcham, A.S., Kasten, B., and Aurbach, G.D.: Intrathyroidal parathyroid adenoma or hyperplasia: an occasionally overlooked cause of surgical failure in primary hyperparathyroidism. J. Am. Med. Assoc. 234: 1029-1033, 1975.

2. Leight, G.S., Parker, G.A., Sears, H.F., Marx, S.J., and Terrill, R.E.: Cryopreservation and autotransplantation of canine parathyroid glands: technique and demonstration of function. Ann. Surg., in press.

Section on Gastroenterology, Digestive Diseases Branch

National Institute of Arthritis, Metabolism, and Digestive Diseases

During this past year most of the laboratory investigations in this section have been directed toward elucidating the biochemical basis of action of gastrointestinal hormones on pancreatic acinar cells.

Dispersed acinar cells prepared from guinea pig pancreas have two functionally distinct classes of plasma membrane receptors, each of which interacts with secretin and with vasoactive intestinal peptide (VIP). One receptor has a high affinity for VIP and a low affinity for secretin. Occupation of this receptor by VIP but not by secretin causes a 3-fold increase in cellular cyclic AMP. The other receptor has a high affinity for secretin, a low affinity for VIP, and occupation of this receptor by either peptide causes a 5- to 9-fold increase in cellular cyclic AMP.

In dispersed pancreatic acinar cells the initial step in the action of cholecystokinin (CCK) and muscarinic cholinergic agents is a 5-fold increase in the rate of calcium outflux. This increased calcium outflux can be accounted for completely by mobilization of membrane-bound calcium and is followed sequentially by a 10- to 14-fold increase in cellular cyclic GMP and a 2- to 3-fold increase in amylase secretion.

Results of our clinical investigations indicate that patients with Zollinger-Ellison syndrome can be managed satisfactorily with oral administration of histamine H_2-blocking agents such as metiamide and cimetidine. Patients with pancreatic cholera syndrome and metastatic pancreatic neoplasm show significant improvement following therapy with streptozotocin. Patients who have the syndrome but who do not have a neoplasm pose particularly difficult diagnostic and therapeutic problems and we are currently attempting to develop a rational approach to managing such patients.

(Drs. J. Gardner, D. McCarthy, E. Olinger, R. May, A. Dubois, H. Shelby, S. Batzri, M. Jackson, L. Sjodin, B. Long and A. Tousimis; and T. Conlon, T. Adams, and A. Childress.)

Serial No. NIAMDD-DDB
1. Digestive Diseases
2. Diseases of the Liver
3. Bethesda, Maryland

PHS-NIH

Annual Report of the Section on Diseases of the Liver, DDB:NIAMDD
July 1, 1975 through June 30, 1976

The Section on Diseases of the Liver is currently conducting five principal studies. One study listed in last year's report, Clinical and Histologic Studies of Vinyl Chloride Associated Liver Disease, has been discontinued because of the unavailability of adequate numbers of patients.

I. Studies of the Transport of Organic Anions by the Liver

A principal effort in this study involves the development of techniques for quantitating individual steps in the hepatic transport of various cholephilic anions, both in man and in animals, by employing kinetic studies with BSP, indocyanine green, cholyl-glycine and radiolabeled bilirubin. By means of kinetic techniques we have previously demonstrated that Gilbert's syndrome represents a heterogeneous condition. In type I, the metabolic defect appears to be restricted to bilirubin metabolism. Type II has, in addition, a defect in hepatic uptake of other cholephilic anions such as BSP and indocyanine green, whereas in type III the initial hepatic uptake of these materials is normal but abnormal hepatic clearance results from an abnormality in later stage in their transhepatic transport. During the past two years investigation of bile acid metabolism in Gilbert's syndrome has demonstrated that both fasting and post-pranial serum bile acid concentration and cholyl-glycine disappearance curves are entirely normal in all three of these subtypes of Gilbert's syndrome. These studies confirm previous information which suggest that the hepatic transport of bile acids occurs over separate pathways from those involved in the transport of other cholephilic anions. This finding is also of clinical significance in that the finding of a normal serum bile acid level or bile acid disappearance curve would mitigate strongly against the need for liver biopsy in patients with an unconjugated hyperbilirubinemia. Additional studies of bilirubin metabolism during the past year have been performed in patients with hereditary spherocytosis, in whom bilirubin clearance was determined before and after splenectomy. In patients whose pre-operative bilirubin clearance values were normal, the fall in bilirubin production resulting from splenectomy had no effect on hepatic bilirubin clearance, whereas in patients whose pre-operative bilirubin clearance values were reduced to the range suggestive of Gilbert's syndrome there was a marked increase in hepatic bilirubin clearance following surgery. This suggests that in patients with Gilbert's syndrome the hepatic bilirubin transport system is operating at a level close to saturation. This observation further suggests that bilirubin loading studies may represent a useful provacative test for the diagnosis of carriers of the Gilbert's syndrome gene who do not express the trait at basal levels of bilirubin production. Animal studies during the past year have concentrated on examining the intra-hepatic metabolism of conjugated bilirubin, employing the new preparation of radiolabeled bilirubin conjugates developed last year in our laboratory. In

contrast to previous data involving crude preparations of bilirubin conjugates, use of purified material has made it possible to demonstrate that conjugated bilirubin in fact has a significant affinity for intracellular anion binding proteins, and that there is an intrahepatic compartment of stored conjugated bilirubin. The demonstration of this compartment requires a re-examination of current concepts of the nature of bilirubin excretion from the liver and future studies within the next year will be directed toward further elucidating the nature of this pathway.

II. The Removal of Organic Anions from Blood by Hemoperfusion

During the past year this project has continued the previous investigation of the effectiveness of albumin-agarose affinity chromatography columns for the removal of bilirubin from the blood of neonatal Rhesus monkeys with either endogenous neonatal hyperbilirubinemia or hyperbilirubinemia resulting from the infusion of large amounts of unconjugated bilirubin. Current studies concentrated both on quantitating the capacity of the columns and on examining further the effects of citrate infusions on preventing entrapment of platelets and granulocytes over the columns. These studies demonstrated that albumin-agarose hemoperfusion represents a highly effective means of reducing the serum bilirubin concentration and further demonstrated that the use of citrate infusions can virtually completely abolish platelet and granulocyte losses during such a procedure. Detailed studies in individual monkeys demonstrated that even very high levels of citrate in blood were well tolerated without manifestations of toxicity provided that adequate quantitites of calcium were added to maintain a normal ionized serum calcium level. These studies, therefore, demonstrate the clinical feasibility of employing citrate to protect platelets and granulocytes during extracorporeal perfusion through a variety of devices.

III. Immunologic Studies in Chronic Hepatitis and Primary Biliary Cirrhosis

The role of abnormal immune mechanisms in the pathogenesis and perpetuation of the hepatobiliary lesions in chronic hepatitis (CH) and primary biliary cirrhosis (PBC) is being studied. Patients selected for study undergo a comprehensive evaluation of their immunologic status, which includes tests of the functional capacity of the afferent (antigen processing) and efferent (cellular and humoral) limbs of the immune response. Using an <u>in vitro</u> assay system which has been shown to be both sensitive and reproducible, the cytotoxic potential of lymphocytes has been studied using two different target cells: the Chang cell, a human liver-derived cell, and the EL-4 mouse sarcoma cell, a non-human, non-hepatic cell. The studies have revealed in patients with PBC, a defect in direct cellular cytotoxicity, which is mediated by the K cell, while another K cell mediated process, antibody-dependent cellular cytotoxicity and T cell mediated mitogen-induced cellular cytotoxicity are normal in this disease. These findings imply that a dissociation exists between direct and antibody-dependent functions of the K cell. The cytotoxic response found in HB_sAg negative CH and PBC does not exhibit specificity for the Chang cell and does not indicate prior

sensitization to antigens on this target cell. Evidence of liver-specific cytotoxic phenomena in PBC and CH is being sought using various liver specific target cells and the behavior of the K cell subpopulation of lymphocytes in these diseases is being assessed using new techniques for the quantitation and functional assay of K cells. Methods are being developed for isolation of pure preparations of hepatocytes from needle biopsies of the liver for use as target cells in cytotoxicity assays.

Studies to compare nonspecific and immunospecific reticuloendothelial clearance capacity have been performed in the same patient populations with PBC and CH and patients with alcoholic cirrhosis. Nonspecific clearance of microaggregated albumin was found to be normal, indicating normal hepatic blood flow in patients with compensated disease. In contrast, the clearance of immunospecific particles has been shown to be abnormal in PBC but not in CH or alcoholic cirrhosis. In particular, the clearance of C3b-coated red cells, which depends on their interaction with specific C3b receptors on Kupffer cells in the liver, is markedly impaired in PBC, while splenic clearance of IgG-coated red cells is either normal or increased in this disease. Thus, an immunospecific defect in reticuloendothelial function exists in PBC.

IV. Studies of Copper Metabolism in Man Using ^{67}Cu and ^{64}Cu

Copper metabolism is being studied in vivo by following the fate of simultaneously administered ^{67}Cu and ^{64}Cu. One of the isotopes is given orally and the other intravenously. Serial measurements are made of radioactivity due to both isotopes in the whole body, blood, urine, feces and, using an external probe, the liver. Data obtained from blood comprize separate determinations of radioactivity in red cells, ceruloplasmin and ceruloplasmin-free plasma. The data obtained are being analyzed by computer in terms of a multicompartmental model of copper metabolism. This approach enables various parameters of copper metabolism to be quantitated, such as the kinetics of intestinal absorption, hepatic uptake, biliary include normal volunteers and patients with diseases associated with markedly increased deposition of copper in the liver, in particular, Wilson's disease and primary biliary cirrhosis. In addition, the possibility that there is an abnormal pattern of radiocopper kinetics, which is characteristic of heterozygotes for Wilson's disease, is being investigated.

V. Studies of the Metabolism of Radioiodinated Alpha-1-Antitrypsin and Desialylated Alpha-1-Antitrypsin in Man.

Homozygous alpha-1-antitrypsin (α_1-AT) deficiency (phenotype PiZZ) may be associated with cholestasis in the neonatal period, cirrhosis, liver cell carcinoma and emphysema. In this condition hepatocytes have been shown to contain globules of α_1AT-like material devoid of sialic acid, and the α_1AT in plasma, which is present in abnormally low concentration, has also been shown to have a reduced content of sialic acid. The aim of these studies is to investigate α_1AT metabolism in normal subjects and patients with hepatic disease and in particular to examine the relationship between the structure of α_1AT and abnormal plasma concentrations of this protein. The studies involve performing metabolic

turnover studies of normal and abnormal α_1AT molecules in normal subjects, patients with aα_1AT deficiency (phenotype PiZZ), relatives of PiZZ individuals with one Z gene and patients with chronic hepatocellular disease unassociated with α_1AT deficiency. Using normal plasma from individuals of phenotype PiMM α_1AT has been isolated and shown to be immunochemically pure and to have retained its characteristic microheterogeneity when subjected to electrophoresis at an acid pH. This material after trace-labeling with radioiodine has been used to study the turnover of α_1AT in normal volunteers. Planned studies include the administration of similar material to patients with α_1AT deficiency and the administration of labeled α_1AT isolated from patients with α_1AT deficiency and labeled desialated α_1AT to both normal subjects and patients with α_1AT deficiency. This protocol will enable the contribution of decreased hepatic release of α_1AT and increased hepatic uptake of α_1AT to the low serum concentration of α_1AT in α_1AT deficiency to be assessed. It will also enable the capacity of the liver in chronic hepatocellular disease to clear an asialoglycoprotein to be determined. Thus, these studies are designed to clarify the mechanism of the low serum α_1AT concentration in α_1AT deficiency and will enable the endogenous catabolism of this protein in man to be related to variations in its molecular structure.

ANNUAL REPORT SUMMARY
DIGESTIVE DISEASES BRANCH

Phoenix Clinical Research Section

In spite of reduced personnel, investigations in to the metabolic problems among southwest American Indians have continued. The major investigation during the past year has focused on low density lipoprotein metabolism in the southwestern Indian. These studies were undertaken because of the rarity of coronary heart disease in the southwest American Indian, their relatively lower values of serum cholesterol and the high prevalence of cholesterol cholelithiasis.

A major finding is that the absolute synthetic rate of apo-B-LDL was significantly lower in the Indians compared to the Caucasians. It was also noted that the Indian subjects had higher absolute HDL cholesterol levels than Caucasians and lower LDL cholesterol levels. These findings suggest that the distribution of cholesterol in the Indians is altered and that the Indians may have a more efficient mechanism for the removal of tissue cholesterol, for example from the arterial wall, than do Caucacians. The findings may also explain the relatively high amount of cholesterol excreted in the bile which leads to the high prevalence of cholesterol cholelithiasis. Studies of HDL and LDL cholesterol are being extended to determine whether or not the alterations in the ratios may explain the infrequent development of coronary heart disease by comparing Indians and Caucasians with and without coronary heart disease and with and without diabetes mellitus.

Studies have been completed on the effect of diabetic control on the parameters of biliary lipid metabolism and cholesterol balance. These studies indicate that control of diabetes reduces the synthesis of cholesterol and reduces the bile acid pool size resulting in an overall increase in biliary lithogenicity in the euglycemic state.

Studies were also completed of the effect of phenobarbital on biliary lipid metabolism. Phenobarbital has been suggested as a possible therapeutic agent for the treatment of gallstones since in the Rhesus monkey it had been shown to reduce biliary lithogenicity. In normal man, however, no changes in any of the parameters of biliary lipid metabolism measured occurred after the administration of phenobarbital. Thus, phenobarbital does not appear to be a useful agent in the prevention or dissolution of cholesterol gallstones in man. A preliminary investigation

of the effects of dietary fiber on biliary lipid composition similarly revealed no changes in the cholesterol composition of gallbladder bile or in bile acid composition. Thus, it is unlikely that any significant beneficial changes in bile composition can be induced by the supplementation of the diet with bran.

The remaining work of the section during the past year has comprised mainly of completing for publication studies initiated and performed for the most part in the prior reporting period.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53001-06 DDB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies of Membrane Function

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

PI:	J. D. Gardner	Chief, Section on Gastroenterology	DDB, NIAMDD
OTHER:	M. Jackson	Guest Worker	DDB, NIAMDD
	L. Sjodin,	Guest Worker	DDB, NIAMDD
	H. Shelby	Guest Worker	DDB, NIAMDD
	S. Batzri	Visiting Associate	DDB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.1	0.7

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

The broad categories which are included in the project are: 1) Characterizing functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) identifying the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) developing techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane; 4) characterizing the mechanism by which the membrane transport of various substrates is altered in certain diseases; and 5) relating these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

During this past year most of our efforts have focused on exploring the functional characteristics of the amino acid transport systems which are stimulated by insulin and on the role of calcium in various secretory processes.

PHS-6040
(12-75)

By increasing cellular calcium in thymic lymphocytes one can abolish the stimulation of amino acid influx caused by cholera toxin, prostaglandin, theophylline, exogenous cyclic AMP or insulin. Ethanol will abolish the stimulation of amino acid influx caused by insulin but does not alter stimulation caused by exogenous or endogenous cyclic AMP.

Publications:

Levy, A. G., Benson, J. W., Hewlett, E. L., Herdt, J. R., Doppman, J. L. and Gordon, R. S., Jr. Saline lavage: A rapid, effective, and acceptable method for cleansing the gastrointestinal tract. Gastroenterology 70: 157-161, 1976.

DeMyer, W. and Gebhard, R. L. Subacute combined degeneration of the spinal cord with cystinuria. Neurology 25: 994-997, 1975.

Batzri, S. and Gardner, J. D.: Amino acid transport in isolated rat thymocytes: Effects of divalent cations and ethanol. J. Biol. Chem. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53002-04 DDB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Gastrointestinal Hormones

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

PI:	J. D. Gardner	Chief, Section on Gastroenterology	DDB, NIAMDD
OTHER:	M. Jackson	Guest Worker	DDB, NIAMDD
	L. Sjodin	Guest Worker	DDB, NIAMDD
	S. Batzri	Visiting Associate	DDB, NIAMDD
	H. Shelby	Guest Worker	DDB, NIAMDD
	E. J. Olinger	Clinical Associate	DDB, NIAMDD
	R. J. May	Clinical Associate	DDB, NIAMDD
	B. W. Long	Clinical Associate	DDB, NIAMDD
	D. M. McCarthy	Visiting Scientist	DDB, NIAMDD
	T. P. Conlon	Chemist	DDB, NIAMDD

COOPERATING UNITS (if any)

Diabetes Branch, NIAMDD
Div. of Cellular Biology, Kennedy Institute for Rheumatology, London, England

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.4	3.3	2.1

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

In vitro systems are being used to study the mechanism of action of gastrin, secretin, cholecystokinin and vasoactive intestinal peptide hormone with their specific membrane receptors.

Clinical investigations are directed toward developing alternative forms of therapy for and elucidating the pathogenesis of disorders characterized by ectopic production of gastrointestinal hormones (e.g., Zollinger-Ellison syndrome and pancreatic cholera).

PHS-6040
(12-75)

In dispersed acinar cells from guinea pig pancreas, VIP and secretin interact with a common membrane receptor, activate adenylate cyclase and increase cellular cyclic AMP. In these same cells cholecystokinin and cholinergic agents cause release of membrane-bound calcium, increased cellular cyclic GMP and stimulation of amylase secretion.

In patients with Zollinger-Ellison syndrome the histamine H_2-blocking agent, Cimetidine, will abolish gastric acid secretion and, as a result, is a therapeutic alternative to total gastrectomy.

Publications:

Klaeveman, H. L., Conlon, T. P. and Gardner, J. D. Effects of gastrointestinal hormones on adenylate cyclase activity in pancreatic exocrine cells. In Thompson, J. C. (Ed.): Gastrointestinal Hormones. Austin, Texas, U. of Texas Press, 1975, pp. 321-344.

Gardner, J. D., Conlon, T. P., Klaeveman, H. L., Adams, T. D. and Ondetti, M. A. Action of cholecystokinin and cholinergic agents on calcium transport in isolated pancreatic acinar cells. J. Clin. Invest. 56: 366-375, 1975.

Gardner, J. D.: Pancreatic cholera. Editorial. Western J. Med. 123: 309-310, 1975.

Gardner, J. D., Conlon, T. P. and Adams, T. D. Cyclic AMP in pancreatic acinar cells: Effects of gastrointestinal hormones. Gastroenterology 70: 29-35, 1976.

Christophe, J. P., Conlon, T. P. and Gardner, J. D. Interaction of porcine vasoactive intestinal peptide with dispersed pancreatic acinar cells from the guinea pig: Binding of radioiodinated VIP. J. Biol. Chem. (In press)

Robberecht, P., Conlon, T. P. and Gardner, J. D. Interaction of porcine vasoactive intestinal peptide with dispersed pancreatic acinar cells from the guinea pig: Structural requirements for effects of VIP and secretin on cellular cyclic AMP. J. Biol. Chem. (In press)

Christophe, J. P., Frandsen, E. K., Conlon, T. P., Krishna, G. and Gardner, J. D. Action of cholecystokinin, cholinergic agents and A-23187 on accumulation of cyclic GMP in dispersed guinea pig pancreatic acinar cells. J. Biol. Chem. (In press)

Olinger, E. J., McCarthy, D. M., Young, R. C. and Gardner, J. D. Hyperhistaminemia and hyperchlorhydria in basophilic granulocytic leukemia. Gastroenterology (In press)

Christophe, J., Frandsen, E., Conlon, T. P., Krishna, G. and Gardner, J. D. Discussion paper on factors regulating cyclic GMP levels in isolated pancreatic acinar cells. In Case, R. M. and Goebell, H. (Eds.): Stimulus-Secretion Coupling in the Gastrointestinal Tract. Baltimore, Md., University Park Press, 1976, pp. 233-235.

Christophe, J., Conlon, T. P., Robberecht, P. and Gardner, J. D. The specific binding and action of vasoactive intestinal peptide (VIP) on isolated pancreatic acinar cells. In Case, R. M. and Goebell, H. (Eds.): Stimulus-Secretion Coupling in the Gastrointestinal Tract. Baltimore, Md., University Park Press, 1976, pp. 377-380.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53003-03 DDB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Gastric Emptying

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

PI:	J. D. Gardner	Chief, Section on Gastroenterology	DDB NIAMDD
OTHER:	A. Dubois	Guest Worker	DDB NIAMDD
	M. Berman	Chief, Lab. of Theoretical Biology	LTB NCI

COOPERATING UNITS (if any)

Laboratory of Theoretical Biology, NCI

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.1	0.2

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

Using a dye dilution technique combined with rapid sampling, gastric emptying and secretion are being evaluated in vivo in man, dogs, and monkeys. Results obtained from such studies are being used to develop a mathematical model of gastric emptying and to explore the role played by gastric emptying in various clinical disorders such as postoperative ileus, Zollinger-Ellison syndrome, and duodenal ulcer.

PHS-6040
(12-75)

Patients with Zollinger-Ellison syndrome have increased fractional rate of gastric emptying both during fasting and following a meal. In contrast, administration of pentagastrin to normal subjects does not alter fractional rate of emptying during fasting and slows the rate of fractional emptying after a meal. These results suggest that the increased rate of fractional gastric emptying seen in patients with Zollinger-Ellison syndrome is not attributable to gastrin but may be due to an undefined neural or humoral factor.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53004-04 DDB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cyclic Nucleotide Mediated Functions

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

PI:	J. D. Gardner	Chief, Section on Gastroenterology	DDB NIAMDD
OTHER:	M. Jackson	Guest Worker	DDB NIAMDD
	L. Sjodin	Guest Worker	DDB NIAMDD
	S. Batzri	Visiting Associate	DDB NIAMDD
	B. W. Long	Clinical Associate	DDB NIAMDD
	E. J. Olinger	Clinical Associate	DDB NIAMDD
	R. J. May	Clinical Associate	DDB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Gastroenterology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.1	0.7

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

In vitro systems are being used to characterize the mechanisms by which cyclic nucleotides alter cell function and to explore the mechanism of action of agents whose effect on cell function is mediated by cellular accumulation of cyclic nucleotides.

PHS-6040

In dispersed acinar cells prepared from guinea pig pancreas the activity of cyclic nucleotide phosphodiesterase is intimately dependent on cellular calcium concentration. By varying the extracellular calcium concentration one can manipulate cellular concentrations of both cyclic AMP and cyclic GMP, and by so doing modify the final hormone response of the tissue.

Publications:

Aurbach, G. D., Spiegel, A. M. and Gardner, J. D. β-Adrenergic receptors, cyclic AMP, and ion transport in the avian erythrocyte. In Drummond, G. I., Greengard, P. and Robison, G. A. (Eds.): Advances in Cyclic Nucleotide Research, Vol. 5, New York, Raven Press, 1975, pp. 117-132.

Aurbach, G. D., Bilezikian, J. P., Klaeveman, H. L. and Gardner, J. D. The beta-adrenergic receptor and cyclic AMP-mediated sodium transport in the avian erythrocyte. In Taylor, S. (Ed.) Endocrinology 1973, London, William Heinemann Medical Books Ltd., 1974, pp. 343-348.

Levy, A. G., Widerlite, L., Schwartz, C. J., Dolin, R., Blacklow, N. R., Gardner, J. D., Kimberg, D. V. and Trier, J. S. Jejunal adenylate cyclase activity in human subjects during viral gastroenteritis. Gastroenterology 70: 321-325, 1976.

Gardner, J. D., Aurbach, G. D., Spiegel, A. M. and Brown, E. M. Receptor function and ion transport in turkey erythrocytes. In Greep, R. O. (Ed.): Recent Progress in Hormone Research, Vol. 32, Proceedings of the 1975 Laurentian Hormone Conference, New York, Academic Press (In press)

Aurbach, G. D., Brown, E. M., Spiegel, A. M. and Gardner, J. D. Beta-adrenergic receptors, cyclic AMP and ion transport in the avian erythrocyte. In Beers, R. F. Jr. and Bassett, E. G. (Eds.): Cell Metabolism Receptors for Viruses, Antigens and Antibodies, Polypeptide Hormones, and Small Molecules. (Proc. of the Ninth Miles International Symposium), New York, Raven Press (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53005-02 DDB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biochemistry of the Small Intestinal Mucosa

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

PI:	D. M. McCarthy	Visiting Scientist	DDB NIAMDD
OTHER:	R. J. May	Clinical Associate	DDB NIAMDD
	J. D. Gardner	Chief, Section on Gastroenterology	DDB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.2	1.3

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

Using in vitro systems, studies are in progress to characterize the subcellular distribution of various membrane-bound enzymes of the intestinal mucosa, as well as their roles in digestive and absorptive function.

PHS-6040
(12-75)

Some investigators have claimed that hexokinase is associated with the brush border membrane of the intestinal epithelial cell. We have found that the presence of this enzyme in brush border preparations is attributable to mitochondrial contamination.

Publications:

Dolin, R., Levy, A. G., Wyatt, R. G., Thornhill, T. S. and Gardner, J. D. Viral gastroenteritis induced by the Hawaii Agent. Jejunal histopathology and serologic response. Am. J. Med. 59, 761-768, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,500-03 DDB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies of the Transport of Organic Anions by the Liver

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

Principal Investigator: Paul D. Berk, M.D.

Other Investigators: Allan W. Wolkoff, M.D., John M. Vierling, M.D., Bennett L. Blitzer, M.D.

COOPERATING UNITS (if any)

None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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

Plasma disappearance curves of radiolabeled bilirubin, sulfobromophthalein, indocyanine green and bile acids are obtained in normal volunteers and in patients with hepatic disease. Curves are analyzed by computer to calculate anion clearance (e.g. C_{BR} [ml/min/kg]; C_{BSP}, etc.) and, in the case of bilirubin, daily bilirubin turnover (BRT [μmol/kg/day]). Data are further analyzed in terms of compartmental models to estimate hepatic uptake and conjugation rates, storage capacity, liver:plasma concentration gradients and other parameters of the hepatic organic anion transport mechanism which are of interest both for physiologic studies and in the diagnostic classification of hepatic dysfunction. Micromodifications of the above techniques have been developed which permit analagous data to be obtained in small animals.

In addition, a technique has been developed for the isolation, purification and radiolabeling of conjugates of bilirubin. Studies with these preparations have indicated an appreciable affinity of conjugated bilirubin for intrahepatic binding proteins, such as ligandin, resulting in the existence of a significant intrahepatic storage pool for conjugated bilirubin. These two observations will require a re-examination of current concepts of the nature of the hepatic excretion of conjugated bilirubin.

Project Description:

Objectives:

1) To study the transport of organic anions from blood through the liver into bile, in order to characterize the biophysical nature of each of the transport steps involved in the overall hepatic excretory system.

2) To develop a capability for quantitating the function of each of these steps in patients with hepatic dysfunction by analysis of kinetic studies of various test substances such as sulfobromophthalein, indocyanine green and radiolabeled bilirubin.

Methods Employed:

Unconjugated bilirubin-^{3}H or -^{14}C is produced biosynthetically in dogs with external biliary fistulae using δ-aminolevulinic acid-^{14}C or -^{3}H as a precursor. After appropriate crystallization and filtration to render the product sterile and pyrogen-free, radiolabeled bilirubin is injected intravenously into patients or animals in order to determine plasma radiobilirubin disappearance curves. Analysis of these curves permits direct calculation of both hepatic bilirbuin clearance (C_{BR}: ml/min/kg) and plasma bilirubin turnover (BRT: μmols/kg/day). Further analysis of the disappearance curves in terms of a previously proposed compartmental model of bilirubin metabolism permits direct calculation of such parameters of physiologic interest as hepatic bilirubin uptake rate, the intrahepatic bilirubin concentration, the liver to plasma concentration gradient and the hepatic bilirubin storage capacity. Similar kinetic analyses of plasma disappearance curves of sulfobromophthalein and indocyanine green are also performed.

Major Findings:

By means of kinetic techniques we have previously demonstrated that Gilbert's syndrome is a heterogeneous condition. In type I the metabolic defect appears to be restricted to bilirubin metabolism. Type II has, in addition, a defect in the hepatic uptake of cholephilic anions such as BSP and indocyanine green, whereas in type III the initial hepatic uptake of these materials is normal but abnormal hepatic clearance results from a defect in a later stage in their transhepatic transport. The presence of an abnormal BSP test in the latter two groups often leads to considerable confusion and frequently necessitates a percutaneous liver biopsy to rule out the existence of structural liver disease. During the past two years investigation of bile acid metabolism in Gilbert's syndrome has demonstrated that fasting and postpranial serum bile acid concentrations and cholylglycine disappearance studies are entirely normal in all three of these subtypes of Gilbert's syndrome. These studies confirm previously fragmentary information which suggests that the transhepatic transport of bile acids occurs over separate pathways from those involved in the excretion of other cholephilic anions. This finding is also of clinical significance in that even minimal structural hepatic disease has been found almost invariably to be associated with an abnormality in either the postpranial bile acid

concentration or plasma bile acid disappearance rates. Hence, in those subgroups of Gilbert's syndrome associated with abnormalities of BSP metabolism the presence of normal bile acid studies strongly mitigates against the existence of structural liver disease and can be used as a means of obviating the necessity for invasive procedures such as liver biopsy in these patients.

Studies of the relationship of the rate of bilirubin production to the efficiency of hepatic bilirubin clearance have been continued using patients with hereditary spherocytosis as the study population. Since splenectomy essentially abolishes the hemolytic anemia in this condition, studies of bilirubin kinetics before and after splenectomy enable the evaluation of hepatic function in a given individual at two different rates of bilirubin production without any other exogenous or extraneous influences such as the administration of drug therapy. To date, plasma bilirubin turnover and hepatic bilirubin clearance have been determined pre- and post-splenectomy in 11 patients with hereditary spherocytosis, in whom a significant hemolytic state was manifested by a pre-operative chromium-51 red cell half life of 12 $\pm$ 2 days. In five of the patients (group A) pre-operative hepatic bilirubin clearance was normal (0.58 $\pm$ 0.10 ml/min/kg) and was unchanged post-operatively despite a marked fall in plasma bilirubin turnover from 18 $\pm$ 5 to down to 5 $\pm$ 2 mg/kg/day. In the other 6 patients (group B) pre-operative hepatic bilirubin clearance was reduced to a range suggestive of Gilbert's syndrome (0.12-0.28 ml/min/kg) but increased to 176 $\pm$ 23% of baseline ($p < .01$) as bilirubin turnover fell from 21 $\pm$ 4 to 6 $\pm$ 1 mg/kg/day post-operatively. In 3 of the 6 patients in group B, post-operative values for hepatic bilirubin clearance were within the normal range and in the remainder they were just below the normal range. Nevertheless, family history and studies of the bilirubin conjugating enzyme, UDP glucuronyl transferase, performed on needle biopsy specimens obtained during surgery, suggested that these patients did, in fact, have Gilbert's syndrome. These studies are compatible with the concept that some aspect of bilirubin transport is more readily saturated by an increased bilirubin load in patients with Gilbert's syndrome than in normal subjects. Furthermore, latent Gilbert's syndrome which is not evident at basal rates of bilirubin production may be unmasked by situations, such as hemolytic states, which present an increased bilirubin load to the liver. These studies may explain why family studies in Gilbert's syndrome which are based solely on measurements of plasma bilirubin concentration almost invariably detect a smaller number of affected family members in a given kindred than would be predicted by the expected autosomal dominant pattern of inheritance. As a prelude to future family studies, we are currently studying the kinetics of an injected load of 5 mg/kg of bilirubin in normal volunteers and patients with each of the three subtypes of Gilbert's syndrome. Preliminary data suggests that in both patients and normal volunteers the hepatic clearance of a 5 mg/kg load of bilirubin is less than that of a tracer dose of radiolabeled material. However, in patients with Gilbert's syndrome the reduction produced by the 5 mg/kg load is proportionally much greater than that observed in normal volunteers. If this observation is confirmed in a larger series, then it may be possible to use the disappearance curve of a 5 mg/kg loading dose of bilirubin as a more effective screening test for latent Gilbert's syndrome in genetic studies.

Previous animal studies based on crude and highly impure preparations of conjugated bilirubin have suggested that bilirubin conjugates did not bind to any significant degree to intracellular binding proteins, such as ligandin. Accordingly, it was widely believed that bilirubin was rapidly excreted from the liver cell almost immediately after conjugation. Recent studies by Dr. William Jakoby (A:LBM) using highly purified preparations of conjugated bilirubin furnished from this department have, in fact, indicated that there is a high affinity of conjugated bilirubin for the various intracellular binding proteins and that the affinity of ligandin for conjugated bilirubin approximates that for the unconjugated pigment. Based on these observations detailed studies of the tranhepatic transport of conjugated bilirubin have been carried out in rats. Although quantitative kinetic analyses of these studies are not yet complete, qualitative examination of the data provides clearcut evidence that there is, in fact, a significant pool of protein bound conjugated bilirubin within the liver cell analagous to the "storage" of unconjugated material bound to similar proteins. Kinetics of the turnover of this conjugated bilirubin pool will be studied within the next year. Preliminary data suggest that the pool consists largely of bilirubin monoglucuronide with a relatively small content of bilirubin diglucuronide. This observation may be of relevance to recently reported observations that bilirubin diglucuronide is not formed by the microsomal enzyme bilirubin glucuronyl transferase but is, in fact, the result of a transesterification reaction between two molecules of bilirubin monoglucuronide which is carried out by an enzyme located in the canalicular membrane of the liver cell.

Significance to Biomedical Research:

Studies of organic anion kinetics in patients with Gilbert's syndrome, Crigler-Najjar syndrome and normal volunteers have led to a marked improvement in our understanding of the excretory function of the liver. In particular, these studies have demonstrated that it is possible to quantitate hepatic function in terms of clearances in the manner analagous to the use of creatinine clearance measurements for quantitating renal function. The ability to accurately measure both bilirubin turnover and bilirubin clearance, the two parameters which determine the plasma bilirubin concentration, has made possible a precise functional classification of all cases of unconjugated hyperbilirubinemia. Studies of the sort described above are also leading both to the development of new clinical methods for assessing the function of individual steps in organic anion transport processes and to an improved understanding of the nature of the defects in a variety of disease states.

Proposed Course:

We plan to continue our evaluation of organic anion transport in patients with Gilbert's syndrome. In particular, new approaches to studies of bilirubin kinetics involving the intravenous injection of various loading doses of bilirubin, in addition to tracer doses, will be employed in an attempt to see whether or not those patients with demonstrated abnormalities in the hepatic uptake of indocyanine green and bromosulfophthalein can also be shown to have a corresponding alteration in the hepatic uptake of bilirubin. In addition, the methods currently used for the studies of unconjugated kinetics

will be extended to permit studies of the fate of conjugated radiolabeled bilirubin. This will permit the application of these isotope techniques to a variety of more common disease states, including cirrhosis and viral hepatitis.

Publications:

1. Berk, P.D., Wolkoff, A.W., and Berlin, N.I.: Inborn errors of bilirubin metabolism. Medical Clinics of North America 59: 803-816, 1975.

2. Martin, J.F., Mikulecky, M., Blaschke, T.F., Waggoner, J.G., Vergalla, J., and Berk, P.D.: Differences between the plasma indocyanine green disappearance rates of normal men and women. Proc. Soc. Exper. Biol. Med. 150: 612-617, 1975.

3. Martin, J.F., Vierling, J.M., Wolkoff, A.W., Scharschmidt, B.F., Vergalla, J., Waggoner, J.G., and Berk, P.D.: Abnormal hepatic transport of indocyanine green in Gilbert's syndrome. Gastroenterology 70: 385-391, 1976.

4. Berk, P.D., Blaschke, T.F., Scharschmidt, B.F., Waggoner, J.G., and Berlin, N.I.: A new approach to quantitation of the various sources of bilirubin in man. J. Lab. Clin. Med. 87: 767-780, 1976.

5. Berk, P.D., Scharschmidt, B.F., Waggoner, J.G., and White, S.C.: The effect of repeated phlebotomy on bilirubin turnover, bilirubin clearance and unconjugated hyperbilirubinemia in the Crigler-Najjar syndrome and the jaundiced Gunn rat: application of computers to experimental design. Clin. Sci. Molec. Med. 50: 333-348, 1976.

6. Scharschmidt, B.F., Waggoner, J.G., and Berk, P.D.: Hepatic organic anion uptake in the rat. In Paumgartner, G. and Preisig, R. (Eds.): The Liver: Quantitative Aspects of Structure and Function, Vol. II, Basel, S. Karger, 1976.

7. Berk, P.D., Martin, J.F., Vierling, J.M., Wolkoff, A.W., Scharschmidt, B.F., Vergalla, J., and Waggoner, J.G.: Heterogeneity of Gilbert's syndrome, as manifested by abnormalities in the hepatic transport of sulfobromophthalein and indocyanine green. In Paumgartner, G. and Preisig, R. (Eds.): The Liver: Quantitative Aspects of Structure and Function, Vol. II, Basel, S. Karger, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,501-03 DDB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Removal of Organic Anions From Blood by Hemoperfusion

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

Principal Investigator: Paul D. Berk, M.D.

Other Investigators: Bennett L. Blitzer, M.D.

COOPERATING UNITS (if any)
Arthritis and Rheumatism Branch, NIAMDD (Dr. Paul H. Plotz); Animal Resources Branch, DRS (Dr. D. Johnson)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1 1/2	1/2

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

By conjugating human albumin to agarose, affinity chromatrography columns have been developed which are highly effective in removing bilirubin from whole blood. Hemoperfusion of jaundiced rats for one hour with such columns has removed virtually the entire circulating bilirubin pool. Studies of this technique in jaundiced newborn monkeys have demonstrated a similar high degree of efficiency for removing bilirubin. These studies demonstrated an interesting species variation in the effects of hemoperfusion on formed element loss. Virtually no granulocytes or platelets were lost during hemoperfusion of rat blood through albumin-agarose but dramatic and clinically significant losses occurred in monkeys. Examination of the effects of calcium chelating agents and various prostaglandins on platelet and granulocyte losses during hemoperfusion have resulted in the development of techniques which completely abolish such losses during perfusion over a variety of sorbents including albumin-agarose gel, activated charcoal and neutral and charged synthetic resins. The focus of this study has now shifted to the development of a reproducible animal model of reversible fulminant hepatic failure. Such a model will be important in studying whether extracorporeal perfusion represents a rational approach to the treatment of fulminant hepatic failure in man.

Project Description:

Objectives:

To develop artificial extracorporeal methods for removing from blood those metabolic products which are ordinarily extracted by the liver. The ultimate objective of this project is the development of an artificial support system for use in the management of hepatic failure.

Methods Employed:

To date we have concentrated on the removal of bilirubin from blood. Bilirubin was selected as a test substance because of its extremely high binding to serum proteins, which makes it particularly difficult substance to remove. We have used the approach of affinity chromatography, using human serum albumin conjugated in high concentration to agarose beads as a prototype of a simple "artificial liver" for the removal of organic anions from the circulation.

Major Findings:

1) Affinity chromatography using albumin-agarose beads has been found to be a highly efficient means of removing bilirubin, BSP, indocyanine green and number of physiologic metabolites from the circulation.

2) In vivo studies in jaundiced rats demonstrated that one hour of hemoperfusion over an appropriate size column was sufficient to remove virtually the entire circulating plasma bilirubin pool. The columns required for those studies were large compared to the blood volume of the animal and required that up to 50% of the animal's blood volume be within the extracorporeal circuit. Since this system was clearly not translatable to clinical use, the extracorporeal apparatus has been redesigned and now consists of four small columns arranged in parallel. Appropriate circuitry permits the perfusion of blood across one column while previously perfused columns are washed with saline, eluted with ethanol and rewashed with saline. The design of this system, therefore, permits sequential perfusion of one column at a time followed by elution and offers an apparatus of virtually infinite bilirubin binding capacity while requiring only 10% of the animal's blood volume to be in the extracorporeal circuit.

Studies employing this newly designed apparatus in the management of the neonatal hyperbilirubinemia seen in premature newborn Rhesus monkeys again demonstrated strikingly effective bilirubin removal. Efficiency of removal was not affected when the hyperbilirubinemia was increased from its endogenous level of approximately 4 mg% up to values as high as 25 mg% by the infusion of large quantities of unconjugated bilirubin intravenously in the newborn period. In contrast to the studies in rats, in which there were no losses of formed elements, hemoperfusion of monkey blood in vivo and human blood in vitro was found to be associated with striking losses of platelets and granulocytes on the columns. A detailed investigation of this effect revealed that a variety of calcium chelating agents, including citrate, oxylate and ethylene diamintetraacetic acid, when added to heparinized blood in vitro, would prevent such

platelet losses over a variety of materials including activated charcoal, XAD resin and a selected group of cation and anion exchange resins. Subsequently this technique was developed for in vivo use. Hemoperfusion of monkeys over albumin-agarose, charcoal or resin columns was accompanied during the first 80 minutes of a 160 minute perfusion by the infusion of citrate anion on the arterial side of the column. Sufficient calcium chloride was infused on the venous side of the column to maintain a normal ionized calcium concentration in the monkey. During the period of time when the citrate and calcium infusions were continued hemoperfusion was associated with no losses of platelets or granulocytes as measured either in the column effluent or within the monkey. Cessation of citrate and calcium infusion was associated with a dramatic fall in platelet and granulocyte counts with platelet counts often reaching levels as low as 10% of baseline. Significant hemorrhagic phenomenon developed in association with these platelet losses. An extensive evaluation of the use of citrate revealed no evidence whatsoever of toxicity from the very high resulting citrate anion concentrations in monkeys provided that normal ionized calcium concentrations are maintained. It is hoped that modifications of this technique will permit the expanded use of not only of albumin-agarose gel in experimental studies but also of charcoal and resin hemoperfusion whose clinical application in third generation artificial kidney devices and in the treatment of drug intoxication has been severely restricted because of thrombocytopenic complications.

A major clinical application of extracoporeal perfusion at the moment is the use of charcoal as a sorbent for the management of acute fulminant hepatic failure in man. Preliminary ethusiastic studies employing this technique suggested that approximately 40% of patients with acute fulminant hepatic failure could be salvaged by this means in contrast to a survival rate of 20% or less during conventional therapy. More recent studies of this treatment have been less enthusiastic and it now appears that the salvage rate is approaching that of conservative management. Little is known about what biochemical alterations in patients with liver failure result from this extracorporeal perfusion. Because of the potential importance of this technique, we have been investigating various animal models of acute fulminant hepatic failure. A commonly used surgical model involving infarction of the liver was evaluated but has been rejected because of its inherent irreversibility. Commonly used chemical models, such as carbon tetrachloride or bromobenzene administration have also been rejected because it was found that the severe renal damage accompanying hepatic injury from these agents markedly complicates the biochemical picture and clouds the interpretation of the results. Galactosamine has been found to produce a highly specific hepatic necrosis which histologically closely resembles fulminant hepatic failure in man. The biochemistry of this lesion has previouly been well worked out by others and we have now established that a dosage regimen employing repeated injections in an inbred strain of rabbits produces death from hepatic failure in approximately 90% of the animals. Furthermore, death is preceded by a period of clinically evident coma of 4-6 hours duration. Current studies are investigating the nature of the biochemical abnormalities associated with coma in these animals. Once the biochemical state has been well worked out, the effects of extracorporeal perfusion over a variety of sorbents including charcoal, XAD resin and albumin-agarose gel, both on survival of the animals and on biochemical abnormalities will be systematically investigated.

Proposed Course:

The effects of albumin-agarose gel hemoperfusion on a variety of metabolites will be investigated in newborn sheep. Although sheep do not get neonatal hyperbilirubinemia, newborn lambs are of approximately the same birth weight as human newborns. In addition, because their size is considerably bigger than that of the animals studied to date, larger volumes of blood samples will make it possible to assess other physiologic affects of hemoperfusion in great detail. In particular, it will be necessary to investigate the role of hemoperfusion on levels of important hormones and on plasma levels of bile acids and free fatty acids. Dr. Paul Plotz (ARB:NIAMDD) has devoted a considerable period of time to developing precise gas/liquid chromatographic techniques for the estimation of individual fatty acids and bile acids in blood and these techniques will be applied to samples obtained from perfused sheep. The effect of perfusion on fatty acids is of particular importance since there is evidence to suggest that fatty acids are a critical energy substrate during the neonatal period.

With regard to the hepatic failure model, an attempt will be made to quantitate both the depth and duration of coma by means of electroencephalographic studies in the intoxicated rabbits. Simultaneously with these studies, investigation of the levels of various cerebral neurotransmitters and false transmitters as well as measurements of a variety of metabolites implicated in the biochemistry of human hepatic coma will be conducted. Ultimately, once the parameters of the galactosamine hepatitis coma model have been determined, the effects of hemoperfusion over sorbents such as albumin-agarose gel, activated charcoal and XAD-2 neutral resin will be investigated in detail.

Publications:

1. Berk, P.D., Martin, J.F., Scharschmidt, B.F., and Plotz, P.H.: Current status of artificial hepatic support systems. In Popper, H. and Schaffner, F. (Eds.): Progress in Liver Disease, 5th edition, New York, Grune and Stratton, 1976.

2. Scharschmidt, B. F.: Approaches to the management of fulminant hepatic failure. Med. Clin. No. Amer. 59: 927-935, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,502-03 DDB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Clinical and Histologic Studies of Vinyl Chloride Associated Liver Disease

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

Principal Investigator: Paul D. Berk, M.D.

Other Investigators: Bennett L. Blitzer, M.D.

COOPERATING UNITS (if any)
Laboratory of Pathology, National Cancer Institute (Dr. L. B. Thomas, Dr. T. Triche); Mount Sinai School of Medicine (Dr. Hans Popper)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

INACTIVE

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,503-02 DDB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Immunologic Studies in Chronic Active Hepatitis and Primary Biliary Cirrhosis

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

Principal Investigator: E. Anthony Jones, M.D.

Other Investigators: Paul D. Berk, M.D., John M. Vierling, M.D.

COOPERATING UNITS (if any)
Immunophysiology Section, Metabolism Branch, NCI (Dr. W. Strober, Dr. D. Nelson); Clinical Immunology Section, Laboratory of Clinical Investigation, NIAID (Dr. M. Frank, Dr. C. Jaffe)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	1	1/2

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

Abnormal immune mechanisms are being studied in patients diagnosed as suffering from HB$_s$Ag negative chronic active hepatitis and primary biliary cirrhosis according to strict clinical, serum biochemical, serum antibody and hepatic histologic criteria. A detailed immunologic evaluation is undertaken, which includes the following techniques: multiple intradermal antigen tests, primary immunization with both protein and polysaccharide antigens, quantitation of T and B cell populations using Rosette tests and immunofluorescence, HLA typing, measurement of serum immunoglobulins and functional assay of serum complement components. The in vitro cytotoxic capacity of lymphocytes and lymphocyte subpopulations (T, K and B cells) against several liver-specific and nonhepatic target cells in tissue culture is being evaluated. In addition, the capacity of the reticuloendothelial system to clear nonspecific and immunospecific particles from the circulation is being assessed. Nonspecific clearance is determined from the rate of clearance of ^{125}I-microaggregated albumin and immunospecific clearance from the fate of ^{51}Cr-labeled autologous red cells coated with a specific IgG or IgM antibody. The results of these studies include the demonstration of defects in direct cellular cytotoxicity and the ability of the reticuloendothelial system to clear immunospecific particles in primary biliary cirrhosis.

Project Description:

Objectives:

To evaluate in detail the immunologic status of patients with chronic active hepatitis and primary biliary cirrhosis with special emphasis on immunologic phenomena currently believed to be of pathogenetic significance in these diseases. The ultimate objective is to identify the major pathogenetic mechanisms in both diseases.

Methods Employed:

Criteria for patient selection include consistent history, physical examination, results of serum biochemical tests, serum non-organ specific antibody titers and hepatic histology. Patients with a history of ingestion of hepatotoxic drugs or whose sera are positive for hepatitis B_s antigen are excluded. The study will be limited to HB_sAg negative subjects until adequate safeguards for personnel handling blood products are developed. A few patients with alcoholic liver disease have been studied to generate control data in a chronic liver disease in which the etiology is not primarily immunologic.

The immunologic evaluation includes the following types of investigations: 1) Humoral and cellular immune responses are being assessed by multiple intradermal antigen tests; primary immunization with keyhole limpet hemocyanin and pneumococcal polysacchride with subsequent keyhole limpet hemocyanin skin testing. Quantitation of T and B cell populations using Rosette tests and immunofluorescence; HLA typing; quantitation of serum concentrations of immunoglobulins; and functional (hemolytic) assay of complement components are also included in the protocol. 2) The cytotoxic capacity of lymphocytes and lymphocyte subpopulations harvested from peripheral blood is assessed in an in vitro cytotoxicity assay, using as target cells Chang human hepatoma cells, mouse EL-4 sarcoma cells, human fetal hepatocytes and human fibroblasts. The assay depends on quantitating ^{51}Cr released from labeled target cells by lymphocytes from normal subjects and patients run in parallel. The technique is used to assay direct cellular cytotoxicity, mitogen induced cellular cytotoxicity (using phytohemagglutinin, concanavalin A and pokeweek mitogen) and antibody dependent cellular cytotoxicity (using a specific Chang cell antibody). 3) The capacity of the reticuloendothelial system to remove from the circulation nonspecific and immunospecific particles is being assessed. Nonspecific clearance is determined from the rate of clearance of a test dose of ^{131}I-microaggregated albumin and hepatic blood flow from the rate of clearance of a tracer dose of this substance. Immunospecific clearance is investigated by following the fate of intravenously administered autologous red cells labeled with ^{51}Cr and coated with a pure IgM or IgG antibody directed against ABO or Rh antigens. Clearance of IgM coated cells occurs largely in the liver and depends on the interaction between C3b deposited on the cell surface and specific C3b receptors on Kupffer cells, while clearance of IgG coated cells occurs largely in the spleen.

Using this protocol it is possible to derive information about the function of both the afferent and efferent limbs of a patient's immune response. The assessment of the afferent limb includes data on humoral

immunity (primary and secondary responses) and cellular immunity, including quantitation and functional assay of T, B and K cells.

Major Findings:

Results to date include the following:

1) The cytotoxic response in patients with primary biliary cirrhosis and HB_sAg negative chronic hepatitis is not specific for the Chang cell and does not indicate prior sensitization *in vivo* to antigens on this target cell.

2) Primary biliary cirrhosis is associated with a selective defect in direct cellular cytotoxicity, which has been shown to be mediated by the K cell. The defect appears to be due to a specific dysfunction of the K cell, as both K cell mediated antibody-dependent and T cell mediated mitogen-induced cellular cytotoxicity are normal in these disease. The findings establish a difference in cytotoxic capacity between primary biliary cirrhosis and HB_sAg negative chronic hepatitis and, in addition, imply that in primary biliary cirrhosis a dissociation exists between the direct and antibody-dependent cytotoxic functions of the K cell.

3) There is a higher incidence of skin test anergy in patients with primary biliary cirrhosis than in patients with chronic active hepatitis and controls. This anergy includes the response to de novo immunization with keyhole limpet hemocyanin.

4) An immunospecific defect in reticuloendothelial function has been demonstrated in primary biliary cirrhosis but not HB_sAg negative chronic hepatitis or alcoholic cirrhosis. In primary biliary cirrhosis clearance of IgM-coated red cells, which is dependent on the integrety of the classical complement pathway, is strikingly diminished, while that of IgG-coated red cells is normal or enhanced and that of nonspecific particles is normal. This clearance defect cannot be explained in terms of inadequate circulating complement components and implies impaired interaction between C3b, which is deposited *in vivo* on the surface of the red cell, and specific C3b receptors on Kupffer cells.

Significance to Biomedical Research:

Eludication of the pathogenetic mechanisms which initiate and sustain the disease process in chronic active hepatitis and primary biliary cirrhosis is required for better diagnostic testing and development of rational therapies. Defined initiating factors exist for those subgroups of chronic active hepatitis which are associated with HB_sAg and which follow the ingestion of certain drugs whereas initiating factors are unknown for the remainder of patients with chronic active hepatitis and all patients with primary biliary cirrhosis. The processes leading to sustained disease activity are unknown in both conditions. Current hypotheses favor immunologic mechanism(s). These hypotheses, in part, stem from the high prevalence of abnormal immunologic phenomena in both diseases. The empiric use of adrenal corticosteroids and other immunosuppressive therapies in these diseases appear to be, at least in part, a reflection of this view. To date, existing specific hypotheses lack

experimental confirmation. In addition, it is not known whether certain immunologic abnormalities found in some subgroups of patients and not in others are epiphenomena or are related to pathogenesis.

The current study is designed to provide a comprehensive immunologic evaluation of selected patients so that the spectrum of immunologic abnormalities associated with primary biliary cirrhosis and HB_sAg negative chronic hepatitis can be defined and the relationship of these abnormalities to clinical and histologic variables can be assessed. In addition, hypotheses of specific pathogenic mechanisms are tested. The significant morbidity and mortality of chronic active hepatitis, the uniform mortality of primary biliary cirrhosis, and the inadequacies of all current therapies in both conditions emphasize the need for information which throws light on the pathogenesis of these diseases.

Proposed Course:

The protocol, as described above, will be continued. Investigations will also be directed toward defining the antigenic specificity of the cell membrane of the Chang cell and other hepatic target cells, developing methods of culturing patients' liver cells obtained by percutaneous biopsy for use as target cells and performing functional assays of lymphocyte subpopulations. If appropriate, T-cell suppressor function will be evaluated in collaboration with workers in the Immunophysiology Section of the Metabolism Branch, National Cancer Institute. If adequate safeguards can be instituted, patients with HB_s antigenemia will be included within the protocol.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,504-02 DDB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies of Copper Metabolism in Man Using ^{67}Cu and ^{64}Cu

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

Principal Investigator: E. Anthony Jones, M.D.

Other Investigators: Paul D. Berk, M.D., John M. Vierling, M.D.

COOPERATING UNITS (if any)
Whole Body Counting Section, Nuclear Medicine Division, CC (Dr. R. Aamodt, Mr. W. Rumble); George Washington University (Dr. S. O'Reilly)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	1	1/2

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

To study copper metabolism in vivo the fate of ^{67}Cu is followed after its intravenous injection or the fate of both ^{67}Cu and ^{64}Cu are followed after giving one of the isotopes intravenously and the other by mouth. Subsequently, measurements are made of radioactivity in the whole body, liver, red cells, ceruloplasmin, ceruloplasmin-free plasma, urine and feces. The data obtained are being analyzed by computer using curve-fitting techniques and a multicompartmental model of copper metabolism. The computer analysis enables various parameters of copper metabolism to be quantitated, in particular the kinetics of intestinal absorption, hepatic uptake, biliary excretion and incorporation into ceruloplasmin. The primary goal of these studies is to obtain an improved understanding of copper metabolism in normal subjects and in patients with diseases associated with increased deposition of copper in the liver, notably Wilson's disease and primary biliary cirrhosis. Another goal is to determine whether a set of abnormalities of radiocopper kinetics can be defined which would enable subjects who are heterozygous for Wilson's disease to be distinguished from those who are homozygous and those who are neither homozygous not heterozygous for this disease.

PHS-6040
(12-75)

Project Description:

Objectives:

To evaluate quantitatively the metabolism of copper in health and specific diseases associated with abnormalities of copper metabolism.

The ultimate goals include a comprehensive understanding of normal copper metabolism, and an evaluation of the significance of specific abnormalities of copper metabolism in patients who have increased deposition of copper in the liver, in particular those with hepatolenticular degeneration (Wilson's disease) and with diseases associated with chronic cholestasis such as primary biliary cirrhosis. In addition, relatives of patients with hepatolenticular degeneration, some of whom are heterozygotes for this condition, are also studied, to determine whether abnormalities of copper metabolism characteristic of the heterozygote state can be defined.

Methods Employed:

1) Studies using ^{67}Cu alone. After the intravenous injection of ^{67}Cu serial measurements are made of whole body radioactivity (using a whole body counter), hepatic radioactivity (using a scintillation probe), and radioactivity in red cells, ceruloplasmin-free plasma, ceruloplasmin, urine and feces. With the exception of hepatic radioactivity, these measurements are made for a period of 13 days. Serum ceruloplasmin-bound ^{67}Cu determination involves the removal of non-ceruloplasmin-bound ^{67}Cu from serum samples by passage of samples down small columns of activated charcoal. Plasma non-ceruloplasmin-bound ^{67}Cu is determined by subtracting radioactivity in ceruloplasmin from that in whole plasma. The curves of total body, hepatic, red cell, plasma ceruloplasmin and plasma non-ceruloplasmin radioactivity are defined and the total excretion of radioactivity in urine and feces calculated.

2) Studies using ^{67}Cu and ^{64}Cu. Studies are also being conduced in which the fate of simultaneously administered ^{67}Cu and ^{64}Cu is followed. One of the isotopes is given orally and the other intravenously. The subsequent protocol is the same as for studies using ^{67}Cu alone except that both ^{67}Cu and ^{64}Cu radioactivity are measured in all experimental specimens. In measuring ^{67}Cu and ^{64}Cu simultaneously an appropriate correction is made for the radioactivity due to ^{64}Cu which cannot be excluded when measuring ^{67}Cu. The data obtained are being analyzed by computer. This enables various parameters of copper metabolism to be quantitated, in particular the kinetics of intestinal absorption, hepatic uptake, biliary excretion and incorporation into ceruloplasmin.

Major Findings:

1) The ranges of whole body ^{67}Cu retention for normals and homozygotes and heterozygotes for hepatolenticular degeneration have been defined.

2) Appreciable incorporation of ^{67}Cu into ceruloplasmin in normals and a similar degree of incorporation in heterozygotes for hepatolenticular

degeneration and in patients with primary biliary cirrhosis have been demonstrated. In contrast, incorporation of ^{67}Cu into ceruloplasmin in homozygotes for hepatolenticular degeneration was minimal.

3) A comprehensive model of copper and ceruloplasmin metabolism has been formulated. This model is being utilized in the computer analysis of experimental data obtained after administering both ^{64}Cu and ^{67}Cu to eight normal subjects and four patients with primary biliary cirrhosis.

Significance to Biomedical Research:

In certain disease states marked variations in the copper content of plasma and various organs are known to occur. However, the mechanisms by which copper homeostasis is maintained in health and disturbed in disease are poorly understood. Increasing attention has been focused on copper metabolism in hepatolenticular degeneration since the refutation of the concept that low ceruloplasmin concentration is primarily responsible for manifestations of the disease. In hepatolenticular degeneration and in diseases which are associated with chronic cholestasis, such as primary biliary cirrhosis, very high concentrations of copper have been demonstrated in the liver. It has been suggested that increased hepatic copper may contribute to tissue injury and that reduction of hepatic copper by copper chelation therapy may improve the prognosis in these diseases. By the use of the double radionuclide technique outlined it is possible to determine whether specific disorders characterized by increased hepatic deposition of copper are associated with particular defects in copper metabolism, such as abnormalities of intestinal absorption, hepatic uptake, biliary secretion and incorporation into ceruloplasmin. The pathogenesis of neither hepatolenticular degeneration nor primary biliary cirrhosis is known, and defects in hepatic handling of copper in these diseases could be either directly related to their pathogenesis or secondary phenomena potentiating tissue injury.

By studying heterozygotes for hepatolenticular degeneration, the effect of the presence of a single gene for this condition on copper metabolism can be defined.

Proposed Course:

To achieve the stated objectives it is necessary to study more normal subjects, patients with hepatolenticular degeneration and their relatives, patients with primary biliary cirrhosis and other patients who may have abnormal copper metabolism.

The protocol, as defined above, will be continued. Initially, emphasis will be placed on studying a sufficient number of volunteers to permit normal ranges for variables of copper metabolism to be established. In homozygotes for hepatolenticular degeneration and patients with primary biliary cirrhosis, analysis of the data will focus on the nature of abnormalities of copper metabolism in relation to the manifestations of the disease, while in heterozygotes for hepatolenticular degeneration analysis of the data will be largely directed towards defining defects in copper metabolism which can readily be measured and which enable the heterozygote state to be distinguished from

normal subjects. Further investigations in man may involve direct analysis of copper excretion in bile using a multiple lumen enteric tube. Specific questions relating to radiocopper kinetics which cannot be answered by studies in humans will be investigated in rats using ^{67}Cu and ^{64}Cu.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,505-01 DDB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies of the Metabolism of Radioiodinated Alpha-1-Antitrypsins and Desialylated Alpha-1-Antitrypsin in Man.

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

Principal Investigator: E. Anthony Jones, M.D.

Other Investigators: Paul D. Berk, M.D., John Vergalla, John M. Vierling, M.D.

COOPERATING UNITS (if any)
Whole Body Counting Section, Nuclear Medicine Division, CC (Dr. R. Aamodt, Mr. W. Rumble)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	1	1/2

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

Highly purified biologically active alpha-1-antitrypsins (α1AT's) are being isolated from plasma using gel exclusion chromatography, Concanavalin A affinity chromatography, and DEAE ion exchange chromatography. Isolated preparations of α1AT, trace-labeled with radioiodine, are being used to obtain data on the turnover of α1AT in normal subjects, patients with α1AT deficiency and their relatives and patients with chronic hepatocellular disease. The protease inhibitor genotypes of subjects donating plasma for isolation of α1AT and subjects undergoing α1AT turnover studies are determined. α1AT's, isolated from normal subjects (genotype PiMM) and from patients with α1AT deficiency (genotype PIZZ) have different degrees of sialylation. Each is labeled with a different isotope of iodine and the metabolism of the two labeled preparations are compared in the same individuals. In addition, the metabolism in vivo of α1AT which has been desialylated in vitro is being studied. This project is designed to determine whether the degree of sialylation of α1AT influences the rate at which this protein is catabolized, to evaluate the factors contributing to the low plasma concentrations of α1AT in α1AT deficiency and to assess the effect of chronic hepatocellular disease on the catabolism and/or hepatic uptake of α1AT and desialylated α1AT.

PHS-6040
(12-75)

Project Description:

Objectives:

Alpha-1-antitrypsin (α1AT) is a plasma glycoprotein which contains sialic acid and is responsible for most of the trypsin inhibitory capacity of plasma. The objectives of this study are to obtain data on the metabolism of α1AT in normal subjects (genotype PiMM), patients with α1AT deficiency (genotype PiZZ), relatives of PiZZ individuals with one Z gene and patients with hepatocellular disease unassociated with α1AT deficiency. The possibility that the degree of sialylation of this protein, which is less in PiZZ than PiMM individuals, influences its metabolism in vivo will be investigated by comparing the metabolism of α1AT's isolated from PiMM and PiZZ individuals in the same subjects and by studying the metabolism of α1AT which has been desialylated in vitro.

Methods Employed:

1) The protease inhibitor genotypes of subjects donating plasma for isolation of α1AT and subjects undergoing studies of α1AT metabolism are determined using acid starch gel electrophoresis followed by crossed immuno-electrophoresis.

2) α1AT is isolated from plasma using gel exclusion chromatography, Concanavalin A affinity chromatography and DEAE ion exchange chromatography.

3) The purity of isolated α1AT is determined using gel electrophoresis, crossed immunoelectrophoresis and isoelectric focusing. Its biological activity is assessed by a kinetic assay of tryptic inhibitory capacity using an artificial substrate.

4) To quantitate the turnover of α1AT in man, pure biologically active α1AT trace labeled with radioiodine is administered intravenously and serial measurements of radioactivity in the whole body, plasma and urine are analyzed.

Major Findings:

1) Isolated (PiMM) α1AT has been shown to be pure and to retain its characteristic electrophoretic microheterogeneity.

2) The half life of radioiodinated human (PiMM) α1AT in the plasma of rabbits varies from 8.7 to 10.4 hours.

3) The half life of radioiodinated (PiMM) α1AT in the plasma of 3 normal subjects varied between 90 and 105 hours.

Significance to Biomedical Research:

Homozygous α1AT deficiency (genotype PiZZ) may be associated with cholestasis in the neonatal period, cirrhosis, liver cell carcinoma and emphysema at a young age. In this condition, hepatocytes have been shown to

contain globules of α1AT-like material which is devoid of sialic acid, and the α1AT in plasma, which is present in abnormally low concentration, has also been shown to have a reduced content of sialic acid. The relationship of the characteristic hepatic globules and the low plasma concentration of α1AT to the hepatic lesions associated with α1AT deficiency is unknown. This study is designed to investigate the metabolism of α1AT in α1AT deficiency. The relationship of the characteristic hepatic globules to the rate at which α1AT is delivered to plasma and to the concentration of α1AT in plasma will be assessed. In addition, the contribution of increased catabolism or hepatic uptake in α1AT to reduced plasma concentrations of α1AT will be determined. The study will establish the effect of variations in the molecular structure of α1AT (e.g. different degrees of sialylation) on its rate of endogenous catabolism or hepatic uptake. In particular, the hypothesis that there are hepatic receptors for exposed galactose residues on desialylated glycoproteins which facilitate the rapid hepatic clearance of these proteins will be tested in man. The effect of chronic hepatocellular disease on the liver's capacity to clear a desialylated glycoprotein will be determined. In addition, the influence of chronic hepatocellular disease per se on the metabolism of α1AT will be studied so that important control data can be generated for the evaluation of α1AT metabolism in patients with α1AT deficiency associated with chronic hepatocellular disease.

Proposed Course:

It is proposed to conduct more studies in normal subjects of the metabolism of α1AT isolated from subjects with the genotype PiMM to determine normal ranges for pool sizes, transfer rates between pools and synthetic and catabolic rates for this protein in man. The half lives of normal human α1AT and desialylated human α1AT will be compared in rabbits, before studying the metabolism of desialylated α1AT in man. It is planned to isolate α1AT from individuals with α1AT deficiency (genotype PiZZ) who do not have associated hepatocellular disease and to utilize such preparations of α1AT in comarative studies of the metabolism of PiMM and PiZZ α1AT's in man.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,001-04 DD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cholesterol and Biliary Lipid Metabolism in Obesity

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

PI: P.H. Bennett, Acting Chief — PCRS, NIAMDD

OTHER: L.J. Bennion, Formerly Clinical Associate — PCRS, NIAMDD

S.M. Grundy, Professor of Medicine
University of California at San Diego

COOPERATING UNITS (if any)

University of California at San Diego

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

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

The effects of obesity and caloric intake on biliary lipid metabolism in man was investigated in a series of 23 obese healthy subjects and a group of 23 non-obese controls matched for age, sex, and race. It was found that bile was significantly more saturated with cholesterol in the obese subjects compared to the non-obese controls. When the obese subjects were subjected to weight reduction, a significant reduction in the cholesterol content of bile was noted but there were no differences in the bile acid or phospholipid output. Through the course of these studies supersaturated bile in the obese could be attributed to a single defect in lipid secretion, namely an excessive output of cholesterol. From these studies it was concluded that obesity was characterized by excessive hepatic secretion of cholesterol which results in supersaturated bile, this defect could be ameliorated by weight reduction.

PHS-6040
(12-75)

Project Description:

Objectives: To determine whether obesity is associated with changes in bile which would predispose to gallstone formation, and if so, to determine the mechanisms responsible for these changes.

Methods Employed: Bile lipid composition, bile lipid secretion rates, bile acid kinetics and pool size, bile acid composition have been, and are being, determined in obese subjects before and after weight reduction.

Major Findings: Weight reduction resulted in significant reduction in the level of saturation of human bile with cholesterol, significant reduction in the rate of secretion of cholesterol and significant reduction in bile acid pool size.

Significance to Biomedical Research and the Program of the Institute: Obesity evidently predisposes to cholesterol gallstone formation through the formation of supersaturated bile. The production of supersaturated bile in obesity is not a consequence of a deficiency of bile acids, but rather of an excessive secretion of cholesterol. Weight reduction results in a decreased level of saturation of bile with cholesterol.

Proposed Course: This study has been discontinued and the findings published.

Publications:

Bennion, L.J. and Grundy, S.M.: Effects of Obesity in Caloric Intake on Biliary Lipid Metabolism in Man. J. Clin. Invest. 56:996-1011, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,003-04 DD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effect of Diabetic Control on Cholesterol Metabolism

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

PI: P.H. Bennett, Acting Chief PCRS, NIAMDD
OTHER: None

COOPERATING UNITS (if any)

University of California at San Diego

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

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

Six diabetic Pima Indians who were not receiving either oral hypoglycemics or insulin were kept in a hyperglycemic state. Metabolic parameters of biliary lipid cholesterol composition and total body cholesterol balance were investigated in this hyperglycemic state. Upon completion the patients were treated for their diabetes with insulin. The repeat studies of biliary cholesterol composition and body cholesterol production were investigated in the euglycemic state. Biliary cholesterol composition and body cholesterol production were determined by previously published methods. The preliminary data at this stage suggests that the synthesis of cholesterol is increased in the uncontrolled diabetic state as compared to the euglycemic state. Bile acid pool size is expanded resulting in decreased saturation of bile with cholesterol. It is proposed that cholesterol synthesis may be increased in the diabetic hyperglycemic state, which may provide an explanation for the increase in atherosclerosis among diabetics.

PHS-6040
(12-75)

Project Description:

Cholesterol balance, bile acid pool size and turnover rate, bile lipid composition and hepatic secretion rates of cholesterol, bile acid and phospholipid are being measured in six Pima Indians with maturity-onset diabetes mellitus and uncontrolled hyperglycemia (blood sugars over 250). These measurements are then repeated after normoglycemia (blood sugars around 100) has been achieved with insulin therapy.

Methods Employed: Cholesterol balance is determined during several weeks of hyperglycemia, and at the end of this period bile studies are performed. After regulation of blood sugar with insulin, the above studies are repeated. Cholesterol balance is determined by chemical methods using non-absorbable markers given by mouth. Fecal steroids are quantitated by gas-liquid-chromatography. Isotope dilution methods are employed in the determination of bile acid pool size and turnover rate. Bile lipid composition is determined by standard chemical procedures on bile obtained by duodenal siphonage.

Major Findings: Preliminary data suggests that the synthesis of cholesterol is increased in the uncontrolled diabetic state (as compared with the normoglycemic state) in these subjects, and that bile acid pool sizes are expanded, resulting in decreased saturation of bile with cholesterol.

Significance to Biomedical Research and the Program of the Institute: The findings suggest that cholesterol synthesis is increased in the diabetic hyperglycemic state. If this conclusion is confirmed, it may provide an explanation for the increase in atherosclerosis among diabetics.

Proposed Course: This investigation has now been completed and the results are being prepared for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,004-04 DD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Critical Micellar Concentration of Human Mixed Biliary Micelles

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

PI: P.H. Bennett, Acting Chief — PCRS, NIAMDD

OTHER: W.C. Duane, Formerly Clinical Associate — PCRS, NIAMDD

COOPERATING UNITS (If any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

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

The formation of bile salt-lecithin mixed micelles serves important functions in solubilizing biliary cholesterol, but it's formation is poorly understood. Formation has been investigated by studying the intermicellar bile salt concentration in equilibrium with the mixed micelle, using equilibrium dialysis, which allows estimation of the critical micellar concentration of the mixed micelle.

PHS-6040
(12-75)

Project Description:

Using equilibrium dialysis the intermicellar bile salt concentrations (IMBC) for taurocholate-lecithin systems was much higher than for taurochenodeoxycholate-lecithin micelles with critical micellar concentrations of 3.0 mM and 0.6mM respectively. These results, in part, explain the increased capability of chenodeoxycholate to dissolve gallstones in man. The project has been terminated.

Publications:

Duane, W.C.: The Intermicellar Bile Salt Concentration in Equilibrium with the Mixed-Micelles of Human Bile. Biochim. Biophys. Acta 398:275-286, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,005-04 DD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Hepatic HMG CoA Reductase in Genetically Obese Rats

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

PI: P.H. Bennett, Acting Chief — PCRS, NIAMDD

OTHER: E. Flock, Visiting Scientist — PCRS, NIAMDD

COOPERATING UNITS (if any)

Center for Disease Control, Phoenix Laboratories

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

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

A method has been devised to measure the content of hepatic HMG CoA reductase in genetically obese rats. Studies measuring the content of this enzyme have been discontinued because of nonavailability of Zucker obese rats.

PHS-6040
(12-75)

Project Description:

HMG CoA reductase in the rate-limiting enzyme in the synthesis of cholesterol and the amount of this enzyme in the liver increases rapidly after feeding. Peak values have been found by others at midnight in rats fed ad lib. The elevated levels of blood cholesterol in genetically obese rats could be due either to increased production of cholesterol or decreased conversion to bile acids.

In a preliminary study of 5 months duration, ending in June 1973, twelve genetically obese rats and twelve of their non-obese siblings were fed chow diet ad lib in a room with a dark period from 4:30 pm to 8:00 am. The rats were killed by decapitation between 10:00 am and 11:00 am and thus presumably during fasting. The livers were assayed for cholesterol content and HMG CoA reductase activity. The concentrations of cholesterol and triglycerides in blood were also measured.

Major Findings: The assay of this enzyme in our hands has been greatly improved during a study of this enzyme in the hamster. It would be worthwhile to complete the study as outlined in the summary of proposed work of June 30, 1974 when time and the availability of the Zucker obese rats permit.

Proposed Course: This study has been discontinued.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,006-04 DD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Alcohol Metabolism in American Indians and Caucasians

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

PI: P.H. Bennett, Acting Chief — PCRS, NIAMDD

OTHER: L.J. Bennion, Formerly Clinical Associate — PCRS, NIAMDD

COOPERATING UNITS (if any)

Indiana University Medical Center

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

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

A study was undertaken to see if there were any genetic or racial differences in the rate of alcohol metabolism between a group of southwestern American Indians and a group of Caucasians. The rate of alcohol metabolism was determined by oral alcohol tolerance tests. Blood alcohol determinations were done by gas-solid chromatography. The major findings from this study included no difference in the rates of alcohol metabolism between the group of southwestern American Indians and Caucasians. This casts doubt on previous reports which state that there were differences in the rate of metabolism of alcohol between Indians and Caucasians.

PHS-6040
(12-75)

Project Description:

Objectives: Recent studies have shown genetic and racial differences in the rate of alcohol metabolism. This project is intended to examine racial variations in alcohol metabolism in Southwestern American Indians and Caucasians.

Methods Employed: The rate of alcohol metabolism was determined by oral alcohol tolerance test. Blood alcohol was determined by gas-solid chromatography.

Major Findings: No significant difference between Caucasians and Indians in the average rate of alcohol metabolism was found.

When the data are analyzed in terms of history of alcohol intake, both Caucasians and Indians show an increase in rate of alcohol metabolism associated with a history of heavy intake.

Significance to Biomedical Research and the Program of the Institute: These studies do not confirm the earlier report of more rapid metabolism of alcohol in Caucasians than in Indians, and therefore, cast doubt on the inference that the high rate of alcoholism among Indians may be related to a low rate of clearance of alcohol from the blood.

Proposed Course: Studies completed and project discontinued.

Publications:

Bennion, L.J. and Li, T.K.: Alcohol Metabolism in American Indians and Whites. N. Engl. J. Med. 294:9-13, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,007-03 DD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effects of Short-Term Fasting on Biliary Lipid Metabolism

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

PI: P.H. Bennett, Acting Chief PCRS, NIAMDD
OTHER: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.2	0.4

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

Biliary lipid metabolism was investigated in a series of 6 subjects and a comparison was made between these kinetics in the fasting state and in the fed state. After ingesting a normal diet the patients had gallbladder bile sampled and biliary lipid turnover studies performed. The subjects were then fasted for a period of 5 to 6 days. After the 4th day of fasting, biliary lipid parameters were again determined. The results in this study include the following: 1) total bile acid pool and cholic acid pool were either slightly reduced or unchanged by fasting, both cholic acid synthesis and the fractional catabolic rate of cholic acid was reduced during the fasting state. Gallbladder bile was rendered a less lithogenic during the fasting state compared to the fed state. There were no changes in bile acid composition either in the fed or fasting state.

PHS-6040
(12-75)

Project Description:

Bile acid and cholesterol metabolism during fasting have been studied in subhuman primates; however, the effects of fasting on sterol metabolism in man remain poorly understood. We have used isotope dilution techniques to measure bile acid synthesis and pool size in the fed state and again after a four-six day fast in six subjects. In addition the lipid composition of gallbladder bile has been examined in these subjects during the fed and the fasted state.

Methods Employed: Isotopic cholic acid was administered orally and gallbladder bile obtained on the four subsequent mornings. After the fourth bile sample, each subject fasted for five-six days. On the evening of the fourth fasting day, cholic acid labelled with a different isotope was administered and gallbladder bile obtained the two subsequent mornings. Total bile acid pool size and cholic acid pool size were calculated from the specific activity in bile obtained the first control day and again the first fasting day. This method of pool size determination has been validated previously in this laboratory and is precise to +2.6%. Cholic acid synthesis and fractional catabolic rates were calculated by standard methods for the fed state. Calculation of these parameters for the fasting state was achieved by assuming a single pool model for cholic acid and rapid changes in synthesis and fractional catabolic rates at the beginning of the fast. Gallbladder bile lipid composition and individual bile acid composition were measured by standard techniques.

Major Findings: Total bile acid pool size and cholic acid pool were either slightly reduced or unchanged by fasting. Cholic acid synthesis was reduced by a factor of three or four in all subjects during fasting. The fractional catabolic rate of cholic acid was reduced by fasting in all subjects. Fasting reduced the molar percent of cholesterol (relative to the solubilizing lipids, bile acids and lecithin) in all but one subject. Individual bile acid composition did not show any consistent change during fasting.

Proposed Course: Investigative work has been completed. Project has been discontinued.

Publications:

Duane, W.S., Ginsberg, R.L., and Bennion, L.J.: Effects of Fasting on Bile Acid Metabolism and Biliary Lipid Composition in Man. J. Lipid Res. (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54,009-03 DD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effect of Phenobarbital Upon Biliary Lipid Metabolism

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

PI: P.H. Bennett, Acting Chief PCRS, NIAMDD
OTHER: None

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.3	0.5

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

Recent investigations have indicated that the use of phenobarbital either along or in conjunction with chenodeoxycholic acid may be beneficial in effecting changes in gallbladder bile and making it less lithogenic. Normal volunteers had biliary lipid parameters and bile lipid composition determined both before and after the administration of phenobarbital. Biliary lipid parameters (hepatic cholesterol secretion, hepatic phospholipid secretion, bile acid secretion, gallbladder bile lipid composition, and the composition of individual bile acid species in gallbladder bile) showed no changes between the period of phenobarbital ingestion compared to the period when phenobarbital was not being ingested. It was concluded that phenobarbital did not significantly alter any parameters of the biliary lipid metabolism and does not appear to be a useful agent when used alone for the prevention or dissolution of cholesterol gallstones in man.

PHS-6040
(12-75)

Project Description:

Recent studies have shown in the primate that phenobarbital, a known potent inducer of liver microsomal enzymes, had the following effects on biliary lipid metabolism and composition: 1) Increased bile flow rates 2) Increased bile salt and phospholipid secretion with a concomitant decrease in cholesterol secretion by the liver. 3) A consequent decreased cholesterol content in bile relative to bile salts and phospholipids. 4) Augmented bile salt synthesis rate and bile salt pool size in animals with intact enterohepatic circulations. 5) Enhancement of the maximal bile salt secretion rate in animals with total biliary fistulae.

Studies in human subjects with cholesterol gallstones have shown that phenobarbital may decrease bile cholesterol content and have a useful synergistic role with chenodeoxycholic acid in the dissolution of cholesterol gallstones.

Methods Employed: Normal volunteers are given a tracer dose of radioactively labelled cholic acid, and total bile acid pool size, catabolic rate of cholic acid, and synthesis of cholic acid are determined from the subsequent fall in specific activity of cholic acid over the next four days. The relative bile composition with respect to cholesterol, bile salts, and phospholipids is also determined. Hourly secretion rates of cholesterol, bile salts, and phospholipids are also determined by the previously described method of Grundy and Metzger. All studies are performed on the same patients before and after the ingestion of phenobarbital for two months at a dose of 3 mg/Kg per day; each patient serves as his own control.

Controls are studied in the same manner with the substitution of a placebo for phenobarbital.

Major Findings: Phenobarbital did not significantly alter any of the biliary lipid metabolism parameters measured.

Significance to Biomedical Research and the Program of the Institute: Phenobarbital, at least when used alone, would not appear to be a useful agent for prevention or dissolution of cholesterol gallstones.

Proposed Course: This investigation has been completed. The project has been discontinued.

Publications:

Ginsberg, R.L. and Garnick, M.B.: The Effect of Phenobarbital on Biliary Lipid Metabolism in Normal Man. Gastroenterology (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,010-03 DD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Hepatic HMG CoA Reductase in Hamsters During Gallstone Formation

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

PI: P.H. Bennett, Acting Chief — PCRS, NIAMDD
OTHER: E. Flock, Visiting Scientist — PCRS, NIAMDD

COOPERATING UNITS (if any)

Center for Disease Control, Phoenix Laboratories

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.3	0.6

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

The purpose of this investigation was to determine the effect of a lithogenic diet (one which is known to cause gallstones in hamsters) on the concentration of HMG CoA reductase, the rate limiting enzyme in the synthesis of cholesterol. Syrian hamsters were either fed a normal diet or a lithogenic diet and then at various periods were sacrificed. The hepatic content of HMG CoA reductase was determined. Cholesterol gallstones were produced in the hamsters fed the lithogenic diet and this was accompanied by an increase in the HMG CoA reductase activity of the liver of sacrificed hamsters. The elevation of this enzyme is indicative of increased cholesterol synthesis as a responsible factor in the genesis of cholesterol gallstones in these hamsters.

7/15

Project Description:

The development of cholesterol gallstones in young hamsters was investigated by feeding these animals a diet known to induce gallstones.

Objectives: The purpose of this investigation was to measure the rate-limiting enzyme for the synthesis of cholesterol, HMG CoA reductase. In addition, the changes in biliary lipid composition induced by such a lithogenic diet were examined.

Methods Employed: Hamsters were fed either a lithogenic diet or a normal chow diet. After varying amounts of time, the animals were sacrificed and the livers and gallbladders removed. The cholesterol content and the content of HMG CoA reductase was determined from liver samples and the composition of biliary lipids was determined from the gallbladder samples.

Major Findings: Cholesterol gallstones were found in 31 of 51 hamsters which had been on the lithogenic diet. None of the hamsters on the chow diet developed gallstones. In the lithogenic treated hamsters, the values of HMG CoA reductase were significantly elevated when compared to the hamsters fed chow diets. The increased HMG CoA reductase activity in lithogenic diet fed hamsters is indicative of increased cholesterol synthesis. This could lead to increased cholesterol secretion in bile and thus to lithogenic bile and finally to gallstone formation.

Proposed Course: This investigation has been completed. The results have recently been submitted for publication. The project has been discontinued.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,011-03 DD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effect of Oral Contraceptives on Biliary Lipid Composition

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

PI: P.H. Bennett, Acting Chief — PCRS, NIAMDD
OTHER: L.J. Bennion, Formerly Clinical Associate — PCRS, NIAMDD
M.B. Garnick, Clinical Associate — PCRS, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.2	0.6

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

Women who take the oral contraceptive medication are known to have an increased incidence of cholesterol cholelithiasis and gallbladder disease. The composition of gallbladder bile was investigated in 22 women during a period of contraceptive usage and during a period without contraceptive usage. Gallbladder bile lipid composition as well as bile acid composition was investigated during these two respective periods. The major findings were an increase in biliary lithogenicity in the women during periods of contraceptive usage. In addition, when oral contraceptives were discontinued, gallbladder bile became significantly less lithogenic. These studies indicate that the potential for cholesterol cholelithiasis is increased in women taking oral contraceptive medications.

PHS-6040
(12-75)

Project Description:

The composition of gallbladder bile in relation to the varying hormonal phases of the normal menstrual cycle has been determined in 6 women through a total of eight cycles. The composition of gallbladder bile in 22 women taking oral contraceptive steroids has been measured, and compared to that when they are not taking oral contraceptives.

Methods Employed: Gallbladder bile is collected by duodenal aspiration following an overnight fast. Gallbladder contraction is stimulated by intraduodenal infusion of amino acids. Lipids in gallbladder bile are measured by standard chemical procedures, and the percent saturation of bile with cholesterol is calculated.

Major Findings: No regular or significant variation of bile composition with the menstrual cycle has been detected; however, the ingestion of exogenous contraceptive steroids results in a regular and statistically significant increase in the degree to which gallbladder bile is saturated with cholesterol in these healthy young women.

Significance to Biomedical Research and the Program of the Institute: These findings suggest that 1) epidemiologic surveys of bile composition can be conducted without regard to the timing of the menstrual cycle, and 2) ingestion of oral contraceptives may increase the risk of developing cholesterol gallstones, and 3) the female preponderance of gallstones may be mediated by an effect of the female sex steroids on the lipid composition of bile.

Proposed Course: These studies have been completed. This investigation has been discontinued.

Publications:

Bennion, L.J., Ginsberg, R.L., Garnick, M.B., and Bennett, P.H.: Effects of Oral Contraceptives on the Gallbladder Bile of Normal Women. N. Engl. J. Med. 294:189-193, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,012-02 DD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effect of Dietary Fiber Upon Biliary Lipid Composition

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

PI: P.H. Bennett, Acting Chief — PCRS, NIAMDD
OTHER: M. Hendrikx, Research Dietitian — PCRS, NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.1	0.2

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

Populations ingesting a high fiber content in their diet are reputed to have a lower incidence of cholesterol cholelithiasis. In order to investigate this, 5 subjects had biliary lipid parameters measured both before and then right after an ingestion of a diet containing large quantities of bran. Gallbladder bile was sampled in a routine manner and lipid composition as well as bile acid composition were determined on samples both before and after bran ingestion. The ingestion of a high bran diet did not induce any changes relative to cholesterol composition of gallbladder bile or in any parameters of bile acid composition. It seems unlikely, therefore that the lowered incidence of cholesterol cholelithiasis in populations eating a large amount of fiber are mediated through changes in biliary cholesterol content.

PHS-6040
(12-75)

Project Description:

Objectives: Recent observations have linked an increased incidence of cholesterol gallstones with the consumption of diets high in refined carbohydrate content and low in bulk constituents. Diets high in bulk (fiber) contents have been associated with higher total bile acid pool sizes in man as well as an increased proportion of chenodeoxycholic acid as compared to diets with high refined sugar content. The purpose of the present study is to investigate the effect of a diet supplemented with a high bran (fiber) content on the cholesterol content of gallbladder bile.

Methods Employed: Normal human subjects will have gallbladder bile collected before and after a 4-8 week period of high fiber (bran) consumption. The bile will be analyzed for the relative proportions of cholesterol, bile acid, and phospholipid content as well as the proportions of individual bile acid species comprising the total bile acid pool.

Major Findings: Five subjects have completed the study and have shown no significant changes in bile lipid composition after the period of bran feeding.

Significance to Biomedical Research and the Program of the Institute: It is unlikely that the lower incidence of cholelithiasis in populations eating large amounts of fiber is attributable to the fiber content of the diet and increasing the fiber content of the diet is not useful for the treatment or prevention of cholelithiasis.

Proposed Course: The investigation has been completed. The project is terminated. The findings have been submitted for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,013-02 DD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effect of Oral Contraceptives on Glucose Tolerance

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

PI:	P.H. Bennett, Acting Chief	PCRS, NIAMDD
OTHER:	S.L. Aronoff, Staff Physician	SFSS, NIAMDD
	P.J. Savage, Staff Physician	SFSS, NIAMDD

COOPERATING UNITS (if any)

Phoenix Indian Medical Center

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	0.3

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

Oral contraceptives were previously shown to induce an abnormal cortisone glucose tolerance in subjects who had otherwise normal oral glucose tolerance tests. To investigate the mechanisms of this change, subjects planning to start or stop oral contraceptives were tested by means of an oral glucose tolerance test and cortisone glucose tolerance test with the measurement of glucose and insulin responses during each test, both before, during and after taking oral contraceptives.

PHS-6040
(12-75)

Project Description:

Objectives: The effect of oral contraceptives on carbohydrate metabolism has stimulated much interest, but in spite of the substantial amount of research the results have been, in some instances, contradictory.

The study was designed to verify and further investigate our observation of an abnormal cortisone glucose tolerance test in subjects on combined oral contraceptives in the presence of normal oral glucose tolerance test.

Methods Employed: Female volunteers (with a negative family history for diabetes) who were planning to start or to stop oral contraceptives were studied. An oral glucose tolerance test and a cortisone glucose tolerance test were performed on each woman while on oral contraceptives and again after stopping them or vice versa.

Major Findings: Although the oral glucose tolerance test was similar in each woman on or off oral contraceptives, plasma glucose levels during the cortisone glucose tolerance test were consistently higher while on oral contraceptives. Serum insulin levels measured during each of the testing periods are presently being analyzed.

Significance to Biomedical Research and the Program of the Institute: This study should clearly delineate the effects of oral contraceptives on glucose and insulin response during the oral glucose tolerance test and the cortisone glucose tolerance test.

Proposed Course: Ten women have been completely tested. The data are being analyzed and prepared for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,014-02 DD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Low Density Lipoprotein Metabolism in American Indians

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

PI: P.H. Bennett, Acting Chief — PCRS, NIAMDD
OTHER: M.B. Garnick, Clinical Associate — PCRS, NIAMDD

COOPERATING UNITS (if any)

University of Pennsylvania

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.5	0.3

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

The prevalence of atherosclerotic cardiovascular disease in southwestern American Indians compared to non-Indian populations is significantly lower. In addition, American Indians have lower values of serum cholesterol compared to non-Indian controls. The metabolism of low density lipoprotein and lipoprotein cholesterol composition were investigated to help characterize these differences. Autologous LDL, radiolabelled in the peptide component, was reinjected into volunteers to study LDL kinetics. There were significant differences in the following parameters between Indians and Caucasian controls. The absolute synthetic rate of apoprotein LDL was significantly lower in the southwestern American Indians. This was accompanied by a lower value of apo-LDL and a lower value of LDL cholesterol in the Indians. In addition, the Indians had significantly higher values of high density lipoprotein cholesterol compared to Caucasian controls. These results provide a possible explanation of the relationship between the lower incidence of coronary artery disease seen amongst southwestern American Indians and may help explain the very high prevalence of cholesterol cholelithiasis in this population.

PHS-6040
(12-75)

Project Description:

Objectives: The extremely low incidence of atherosclerotic cardiovascular disease (ASCVD) in southwestern American Indians compared to non-Indian populations and the finding that patients with type II hyperlipoproteinemia have a prolonged biological half-life (T 1/2) and decreased fractional catabolic rate (FCR) of low density lipoprotein (LDL) have stimulated interest in this protein's metabolism in full-blooded southwestern American Indians. Although the incidence of obesity and diabetes mellitus are greatly increased in some Indian populations, the American Indian is able to maintain a lower serum cholesterol than that of the general population. We are studying the turnover rate of LDL to elucidate the significance of this factor and its potential role in exerting a protective effect in predisposing to ASCVD.

Methods Employed:

1. Patients are initially placed on a low cholesterol, high polyunsaturate: saturated fat diet for three weeks.
2. LDL is isolated from plasma via multiple step, density grandient ultracentrifugation.
3. The LDL is iodinated with ^{125}I via the iodine monochloride method.
4. The preparation is tested for immunologic purity (agarose gel electrophoresis) pyrogens and sterility according to U.S.P. standards.
5. The radio-labelled preparation is injected intravenously; plasma decay curves and urinary excretion of radioactivity are measured daily to determine the T 1/2 and FCR, LDL synthetic rate.

Major Findings: Ten full-blooded southwestern American Indians and five Caucasians have completed these studies. The major significant differences have been in the following parameters: The absolute synthetic rate of apo-LDL was significantly lower in the Indians compared to Caucasians. In addition, the value of apo-LDL and LDL cholesterol were significantly lower in the Indians. The Indians also had a higher value of HDL cholesterol compared to Caucasians.

Significance to Biomedical Research and the Program of the Institute: These findings help explain the relationship between the low prevalence of coronary artery disease in the southwestern American Indians. The differences in lipoprotein metabolism may explain both the racial differences in atherosclerosis as well as an increased incidence in cholesterol cholelithiasis.

Proposed Course: This study has been completed and the results are being prepared for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,015-02 DD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Age of Onset of Lithogenic Bile in Southwest American Indians

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

PI: P.H. Bennett, Acting Chief — PCRS, NIAMDD

OTHER: M.B. Garnick, Clinical Associate — PCRS, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

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

The lipid composition of gallbladder bile, bile acid pool size and sex hormone levels will be determined in Pima Indians below 21 years of age to determine the age at which Pima Indian bile becomes lithogenic. These data will indicate the approximate duration of bile lithogenicity prior to the appearance of cholesterol gallstones and indicate the period of life at which prophylactic measures against gallstone formation may be taken. Bile will be obtained by duodenal siphonage after stimulation of gallbladder contraction and bile acid pool size will be measured to determine the ratio of deuterated chenodeoxycholic acid to native chenodeoxycholic acid in bile twelve hours after the intragastric administration of the deuterated form. The observed changes will be correlated with age, level of sex hormones, pubertal history, body size and sex.

PHS-6040
(12-75)

Project Description:

Objectives: The lipid composition of gallbladder bile, bile acid pool size and blood sex hormone levels will be determined in Pima Indians of each sex below 21 years of age.

Methods Employed: Bile will be obtained by duodenal siphonage after stimulation of gallbladder contraction by intraduodenal infusion of amino acids. Bile acid pool size will be measured by determining the mass ratio of deuterated chenodeoxycholic acid to native chenodeoxycholic acid in the bile twelve hours after the intragastric administration of dideutero-chenodeoxycholic acid. Determination of bile acid composition by gas-liquid chromatography will permit calculation of total bile acid pool size from the chenodeoxycholic acid pool size determined as above by mass-spectrometry. Bile lipid composition will be determined and expressed as percent saturation with cholesterol. Changes in bile composition and bile acid pool size will be correlated with changes in blood level of sex hormones, age, pubertal history, body size, and sex.

Major Findings: None

Significance to Biomedical Research and the Program of the Institute: These studies should disclose whether the secretion of abnormally saturated bile is acquired in the pubertal or teen-age period among Pima Indians, or whether it is present in childhood. They should also disclose the relationship between the onset of lithogenic bile and the incidence of cholelithiasis, since the incidence of the latter has been carefully determined in this population. They should also elucidate the role of the sex hormones in the production of lithogenic bile, as well as the importance of bile acid pool size in the production of lithogenic bile in this population. Comparison of body size measurements and of age to bile acid pool size may yield important data regarding the regulation of bile acid pool size.

Proposed Course: The project will be started after July 1, 1976.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54,016-01 DD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

High Density Lipoprotein Cholesterol in Indians and Caucasians

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

PI:	P.H. Bennett, Acting Chief	PCRS, NIAMDD
OTHER:	M.B. Garnick, Clinical Associate	PCRS, NIAMDD
	P.J. Savage, Staff Physician	SFSS, NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.3	0.4

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

Recent studies have implicated the importance of high density lipoprotein cholesterol as being an independent risk factor in the genesis of atherosclerosis. Studies have shown that individuals with a low amount of high density lipoprotein cholesterol tend to be at more risk for the development of ischemic heart disease and myocardial infarction. We are studying the effect of high density lipoprotein cholesterol in a group of normal Indians and Caucasians as well as in a group of diabetic Indians and Caucasians, with and without coronary artery disease. From this study we will attempt to delineate the importance of high density lipoprotein cholesterol values in the pathogenesis of ischemic heart disease.

PHS-6040
(12-75)

Project Description:

Objectives: High density lipoprotein cholesterol will be determined in a series of normal Caucasians and Indians and diabetic Indians and Caucasians with and without coronary artery disease. Measurement of HDL and LDL cholesterol in the above mentioned groups will help elucidate the role of these particular lipoproteins in the genesis of ischemic vascular disease, both in diabetic and nondiabetic patients. High density lipoprotein cholesterol will be determined by ultracentrifugation methods. Patients will be admitted to the Phoenix Clinical Research Section. During this period lipid values will be determined from these patients while consuming a normal standardized U.S. diet.

Major Findings: None

Significance to Biomedical Research and the Program of the Institute: This study should disclose whether high density lipoprotein plays a role in the genesis of atherosclerosis in patients with and without diabetes. It is known for example, that Southwestern American Indians, who have a very low prevalence of coronary disease, have much higher levels of high density lipoprotein cholesterol than do Caucasians. We are trying to expand these findings further to help elucidate the relationship of high density lipoprotein to atherosclerosis.

Proposed Course: We plan to study 10 to 15 individuals in each of the following categories - normal Caucasians, normal Indians, diabetic Indians without heart disease, diabetic Indians with heart disease, diabetic Caucasians without heart disease, and diabetic Caucasians with heart disease.

Publications: None

ANNUAL REPORT - 1975-1976

CLINICAL ENDOCRINOLOGY BRANCH

Dr. Jan Wolff received The Eli Lilly Award of The Endocrine Society, and delivered the Eli Lilly Lecture at the national meeting of the Society in 1976. Dr. Bruce Weintraub was honored by election to the American Association of Clinical Investigation. Two CEB members were guests in foreign research laboratories for extended periods - Dr. Saul Rosen at the National Institute for Medical Research, Mill Hill, and Dr. Harold Edelhoch at Hopital Bicetre in Paris. Several foreign scientists did research in CEB, representing Belgium, France, India, Italy and Japan.

I. Thyroid Biochemistry

A. Thyroid Cell Membrane and the Mechanism of TSH Action

Polycations of many kinds (e.g., ribonuclease, spermine and polylysine) have been found to alter the activity of the adenyl cyclase in bovine thyroid cell membranes. At low concentrations, they enhance both basal and TSH-stimulated cyclase activity. High concentrations of polycations, and polyanions at low or high concentration, inhibit the activity. The effect appears to be a charge-charge interaction which alters the conformation of the plasma membrane but is not restricted to a particular receptor domain. Thus, the membrane conformation is important for the transmission of the signal from the stimulator (e.g. TSH), bound to its receptor, to the adenyl cyclase effector system. Similar effects have been demonstrated with membranes of adrenal and testis (Leydig tumor) cells. (Wolff, Asbury)

Thyroid membrane adenyl cyclase has been solubilized, and preliminary work has shown that the soluble enzyme differs in certain catalytic properties from the *in situ* enzyme. Model adenyl cyclase studies have also been carried out with a bacterium, *Bordetella pertussis*. A soluble cyclase has been purified from the culture medium. It has a molecular weight of ∿70,000 and differs in its catalytic porperties from the previously described bacterial cyclase. This organism has been shown to have adenyl cyclase activity in four compartments: 1) in the culture medium 2) associated with intact cells and measured with exogenous ATP, 3) extracytoplasmic but sensitive to trypsin, 4) intracellular. Commerical pertussis vaccines contain adenyl cyclase activity which probably is derived from the extracytoplasmic compartment. (Wolff, Hewlett)

Cyclic AMP resulting from the activation of thyroid adenyl cyclase by TSH appears to exert its effect on the thyroid cell by activating protein kinase which then phosphorylates one or more unidentified protein substrates. The subsequent step in this control system, dephosphorylation, has been investigated. A phosphoprotein phosphatase has been found in bovine thyroid tissue, and has been purified extensively. The kinetic and cofactor requirements of this enzyme

has also been demonstrated in normal placenta, and its characterization is now in progress. (Rall, McClung)

B. Iodination Reactions

A key function of the thyroid gland is to iodinate tyrosine residues in thyroglobulin, leading to hormone synthesis. The enzyme which carries out this reaction is thyroid peroxidase. Studies are in progress on the thyroid enzyme, and also on lactoperoxidase and horseradish peroxidase. These three enzymes have similar properties, but the latter two are used as models because of their solubility. Sulfhydryl compounds (e.g., cysteine) inhibit peroxidase by reacting with the heme iron. Diitotyrosine binds to the enzyme at another site, but requires sulfhydryl. These properties have been utilized for affinity chromatography. Glutathione or diiodotyrosine bound to agarose have been used to specifically adsorb each of the three peroxidases, and purification of lactoperoxidase has been achieved. (Cahnmann, Pommier)

C. Iodoproteins

Further study of the polypeptide chain of thyroglobulin has been carried out in guinea pigs. Previously it was shown in this species that reduction and alkylation of 19S thyroglobulin gave three components: A, MW = 295,000; B, MW = 210,000 and C, MW = 110,000. In vivo incorporation of ^{3}H-leucine into these polypeptides showed rapid incorporation into component A, and slower incorporation into B and C. Blocking iodination with methimazole did not alter the kinetics of ^{3}H-leucine incorporation. These studies indicate that the A component is the polypeptide chain synthesized by the thyroid cell. The smaller chains appear to be derived from an in vivo process unrelated to iodination. (Rall, Burkhardt)

The abnormal thyroglobulin previously identified in a rat thyroid tumor (Wollman line 1-8) has been further characterized. The major component (Peak A) was shown not to be an aggregate despite its exclusion from gels with very large pore size. It was shown by equilibrium sedimentation in CsCl gradients to have an unusually high density ($\sim$1.4). Since exposure to crude glycosidase lowered the density to 1.3, this protein appears to have a very high content of carbohydrate. Neither component (Peak A or B) contained thyroxine or triiodothyronine, suggesting that the abnormal structure of these thyroglobulins might prevent iodotyrosine coupling to form the thyroid hormones. (Robbins, Cahnmann, Izumi)

D. Thyroxine Synthesis

Tyrosine polymers have been employed as models for thyroglobulin in the intramolecular coupling of diiodotyrosine to form thyroxine. Linear random copolymers of lysine and tyrosine or glutamic acid and tyrosine give thyroxine when exposed to thyroid peroxidase, iodide and hydrogen peroxide. The highest yield is obtained with the Lys-Tyr copolymer. Lactoperoxidase is almost as effective as thyroid peroxidase, but horseradish peroxidase gives much lower iodination and thyroxine synthesis. A number of other polymers, as well as di- and tripeptides of tyrosine, failed to yield thyroxine. (Cahnmann, Pommier, Nunez)

E. Thyroid Hormone Secretion

Further studies have been done with tubulin, the subunit of the microtubules, which are intimately involved in the process of endocytosis in the thyroid cell. The binding of antimitotic agents has been examined in detail. Colchicine binding is enhanced by various inorganic and organic anions, which appear to have a local effect at or near the binding site. Podophyllotoxin and colchicine binding are mutually competitive, apparently related to the trimethoxyphenyl ring present in each compound. The evidence indicates that both podophyllotoxin and colchicine have at least two points of attachment to tubulin, and that they share one of these. (Wolff, Bhattacharyya, Cortese)

F. Thyroxine-Protein Interactions

Affinity labeling of the thyroxine (T_4) binding site of human serum prealbumin has now been accomplished with ^{14}C-labeled bromoacetyl-T_4. Two labeled compounds were identified after acid hydrolysis, iminodiacetic acid, derived from the N-terminal glycine-1, and N^{ε}-carboxymethyl lysine, derived from Lys-9 and Lys-15. The distribution of radioactivity in Gly-1, Lys-9 and Lys-15 was 30:61:9. Since these residues are located near the entrance of the channel in the prealbumin molecule which is the site of T_4-binding, it appears that T_4 is oriented with the phenolic ring near the center of the protein and the side chain near the periphery. (Cahnmann, Robbins, Cheng)

The major T_4 transport protein, thyroxine binding globulin (TBG) has been purified from human serum, and studies of its physical properties and binding site have been initiated. The protein has a molecular weight near 50,000, a single binding site for T_4, and appears to consist of a single polypeptide chain. Bromoacetyl-T_4 and T_4 compete for the same binding site; hence, the former can be used for affinity labeling. (Cahnmann, Robbins, Edelhoch, Cheng, Gershengorn, Lippoldt)

Studies have been undertaken to identify the locus of TBG synthesis and to investigate the hormonal control of this process. Isolated monkey hepatocytes were obtained by *in situ* perfusion with collagenase, hyaluronidase and EDTA. TBG synthesis in these cells was shown by incorporation of ^{14}C-leucine into immunoprecipitable TBG, and secretion was shown by accumulation of labeled TBG in the incubation medium. Using quantitative radioimmunoassay, it was shown that TBG production by hepatocytes obtained from estrogen-treated monkeys was 2.6 fold greater than normal. *In vivo* metabolism of ^{125}I-labeled TBG was also studied in normal and estrogen-treated monkeys, and showed a 3.2 fold increase in TBG production rate. A monkey hepatocarcinoma cell culture was investigated as a possible tool for studying the control of TBG synthesis. These cells secreted TBG into the culture medium, and this was enhanced by addition of physiological concentrations of T_4 (10^{-14} to 10^{-10}M). This is one of few *in vitro* systems responding to such low concentrations of thyroid hormone. (Robbins, Gershengorn, Glinoer)

G. Studies in Thyroid Disease

Thyrotropin releasing hormone (TRH) was administered in large oral doses (200 mg per day) to patients with thyroid carcinoma to test its ability to stimulate tumor uptake. TRH had a persistant stimulatory effect on TSH secretion for 14 days. Stimulation of radioiodine uptake by tumor was more effective with oral TRH than with the customary injection of bovine TSH. Comparison of TRH stimulation with the response to hypothyroidism alone is under study. (Robbins, Weintraub, Gershengorn)

Medullary thyroid carcinoma contains a high histaminase level which can be measured with a sensitive assay. Fine needle aspiration showed elevated histaminase, as well as cytological findings characteristic of this type of tumor, and provided a definite preoperative diagnosis. Further experience with fine needle aspiration of a large number of thyroid nodules is now being evaluated. (Robbins, Weintraub, Gershengorn, McClung)

Increased TSH secretion is a rare cause of hyperthyroidism in man. An 18 year old woman, who did not have a pituitary adenoma, was shown to have persistently elevated serum TSH in the face of hyperthyroid T_4 and T_3 levels. This new syndrome appears to be one of selective partial resistance of the pituitary thyrotrophs to the action of thyroid hormones. In a study of 8 patients with TSH-induced hyperthyroidism, 4 were secondary to pituitary chromophobe adenoma, 3 to selective pituitary resistance to thyroid hormone, and one to generalized resistance to thyroid hormone. (Weintraub, Gershengorn)

II. Polypeptide Hormones

A. Structure

The interaction of the α and β subunits of human chorionic gonadotropin has been analyzed by studying the kinetics of recombination. It has been shown that the subunits recombine by a bimolecular reaction to give an intermediate which then rearranges to give the native hormone. Direct evidence for such an intermediate was obtained by demonstrating that physical combination precedes the appearance of receptor-binding activity. (Edelhoch, Weintraub, Ingham)

B. Secretion and Metabolism

Utilizing antisera specific for the α and β subunits of the glycoprotein hormones it has been shown that secretion of free subunits is increased in primary hypothyroidism (α and TSH-β) and hypogonadism (α and LH-β). The production rate of the α subunit is higher, and it is exceedingly high in about 1/10 of 60 pituitary tumors which have been examined. In contrast to normal pituitary, the tumors do not respond to hypothalamic releasing hormones. Alpha subunit has also been found in cerebrospinal fluid in cases of pituitary tumor with suprasellar extension. Subunit measurement can be used as a diagnostic tool for detection of pituitary tumors. (Weintraub, Rosen)

Normal pituitary explants and mouse thyrotropic tumors are being used to study the regulation of TSH biosynthesis and secretion. Whereas normal pituitary secretes subunits as well as intact TSH and responds to TRH, the tumor secretes a large excess of α subunit and is less responsive to TRH. (Weintraub, Gershengorn, Blackman)

C. Ectopic Protein Production by Tumor

Placental proteins - chorionic gonadotropin and its subunits (hCG, hCG-α, hCG-β), and placental alkaline phosphatase - previously found in 6% of patients with lung cancer, have been found in a comparable proportion of patients with breast and pancreatic cancer. In contrast, 50% of patients with islet cell carcinoma have these marker proteins in blood. Since they are not found in islet cell adenoma, these measurements have diagnostic utility. (Rosen, Weintraub)

hCG and its subunits have been found as secretory products of HeLa cells. This is the oldest permanent human cell line in culture, and is widely used in cell biology. High molecular weight forms of hCG-α have been purified, and studies are in progress to determine whether these are precursors of normal hCG. (Rosen, Weintraub, Lieblich)

III Adrenal and Testicular Cell Secretion

Adrenal and testicular tumors have been grown in continuous cell culture and used to study the control of steroid hormone secretion. A number of compounds containing adenine, including ATP, ADP, AMP, NAD, FAD and adenosine, cause steroidogenesis, but adenine itself does not. The stimulatory effect is comparable to that of ACTH in the immediate time of onset and the magnitude of the effect. Since the response to adenine was enhanced by dipyridamol, an inhibitor of adenosine transport, it appears that adenosine acts on the cell surface. Adenosine stimulates membrane adenyl cyclase in a manner similar to ACTH, but unlike its effect on steroidogenesis, the effect on adenyl cyclase is only partial. (Wolff)

Lithium ion, an inhibitor of thyroid cell secretion, has been shown to inhibit ACTH-stimulated steroid secretion by adrenal tumor cells. This effect appears to be mediated by the stabilization of microtubules. (Wolff)

IV Protein Structure

A comprehensive thermodynamic analysis of the forces responsible for noncovalent interactions in biological molecules has been made. The folding of proteins, nucleic acids and membranes can be understood in terms of interactions with water. This has been shown by the relation of heat capacity to temperature for a large number of surfactant molecules and nucleic acids. Thus it is evident that water plays a fundamental role in stabilizing the structures of many biological macromolecules. (Edelhoch, Osborne)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45000-09 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Thyroxine-Protein Interactions

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

PI:	S.-Y. Cheng	Staff Fellow	CEB NIAMDD
	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD
	H. Edelhoch	Senior Scientist	CEB NIAMDD
	J. Robbins	Chief, Clin. Endo. Br.	CEB NIAMDD
	M. Gershengorn	Clinical Associate	CEB NIAMDD
OTHER:	R. Lippoldt	Sr. Health Services Officer	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.3	.2

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

L-Thyroxine (T_4) and bromoacetyl-L-thyroxine ($BrAcT_4$) bind competitively to human serum prealbumin (PA). [^{14}C]-Labeled $BrAcT_4$ was, therefore, used to characterize the T_4-binding site on PA by affinity labeling. Various degradation and fractionation methods permitted the determination of the points of attachment of the ligand in affinity-labeled PA. Gly-1, Lys-9, and Lys-15 had been labeled in a 30:61:9 ratio.

Thyroxine-binding globulin (TBG) was purified from human plasma. Its amino acid composition was determined. Leu (1 mol/mol TBG) was found to be the carboxy- terminal amino acid. Thus, as well as peptide mapping of a tryptic digest did not support a subunit structure recently claimed for TBG. Competitive binding of T_4 and $BrAcT_4$ to PA as well as binding of T_4 to PA and to $BrAcT_4$-treated PA indicate that the two ligands compete for the same binding site on PA.

Project No. Z01 AM 45000-09 CEB

Project Description:

Work on the affinity labeling of human serum prealbumin (PA) with 2-[^{14}C]-bromoacetyl-L-T_4 ($BrAcT_4$) was continued. Acid hydrolysis of the labeled protein containing 1 mol ligand/mol PA gave two labeled compounds, N^{ϵ}-carboxymethyl-Lys and iminodiacetic acid in a 7:3 ratio. These were derived from N^{ϵ}-labeled Lys and from N-labeled Gly, respectively. The latter was obviously derived from Gly-1, but the origin of the CM-Lys had to be investigated.

Of the eight Lys residues in PA, five are in an unlabeled tryptic peptide and therefore excluded from consideration. In addition, the penultimate Lys next to the carboxyl terminus was excluded since yeast protease C (carboxypeptidase Y) which released Lys-126 quantitatively from labeled PA, did not release any radioactivity. Both of the remaining two Lys residues (9 and 15) were labeled in a 10:1 ratio as shown by a combination of analytical procedures (sequential Edman degradations of labeled PA and of its CNBr digest after fractionation by gel filtration, and treatment of the tryptic digest of maleylated labeled PA with yeast protease C). Thus, the distribution of the radioactivity in the labeled PA was Gly-1; Lys-9; Lys-15 = 30:61:9.

Preliminary work was done on the structure of thyroxine-binding globulin (TBG), the major thyroxine-transport protein of serum, and on the affinity labeling of its thyroxine (T_4) binding site with $BrAcT_4$. TBG was purified from large volumes of human plasma (yield ∿50 mg TBG/10 l plasma) by affinity chromatography on T_4-sepharose followed by DEAE sephadex chromatography and gel filtration.

The amino acid composition of TBG was determined. It differed considerably from some of the literature data. Peptide mapping after tryptic digestion did not indicate a subunit structure of TBG. Determination of the carboxy terminus with carboxypeptidases A, B, and Y (yeast protease C) gave 1 mol Leu/mol TBG. Molecular weight was determined by sedimentation equilibrium on native TBG and on reduced, alkylated TBG in 6M guanidine at neutral or acid pH. All preparations had MW values near 50,000. Our data, therefore, do not support recent reports in the literature that TBG is a tetramer with 4 identical subunits.

Labeling of TBG with a 2.5 fold molar excess of $BrAcT_4$ resulted in the covalent binding of ∿0.2 mol of ligand. Binding of $BrAcT_4$ to TBG was inhibited in the presence of T_4. Equilibrium dialysis experiments showed that TBG labeled with $BrAcT_4$ binds less T_4 than native TBG. Thus, T_4 and $BrAcT_4$ seem to compete for the same binding site.

Publications:

1. Cheng, S.-Y., Cahnmann, H. J. and Wilchek, M.: in Thyroid Research. Robbins, J. and Braverman, L. E. (eds.) Proceedings of the Seventh International Thyroid Conference, Boston, MA, June 9-13, 1975, American Elsevier Publishing Co., New York, p. 294.

2. Cheng, S.-Y., Cahnmann, H. J., Wilchek, M.: Affinity labeling of the thyroxine binding domain of human serum prealbumin with dansyl chloride. Biochemistry 14: 4132-4136.

3. Gershengorn, M. C., Larsen, R. P. and Robbins, J.: Radioimmunoassay for thyroxine binding globulin in human serum. Application to hepatocellular carcinoma. J. Clin. Endo. Metab. 42: 899-905, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45002-02 CEB

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Nonenzymic Model Reactions for the Conversion of 4-Hydroxyphenylpyruvic Acid to Homogentesic Acid

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

PI:	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD
OTHER:	T. Matsuura	Professor	Kyoto University
	I. Saito	Research Chemist	Kyoto University
	M. Yamane	Research Chemist	Kyoto University
	H. Shimazu	Research Chemist	Kyoto University

COOPERATING UNITS (if any)

Department of Synthetic Chemistry, Faculty of Engineering, Kyoto University, Kyoto, Japan

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.2	.1	.1

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

The dye-sensitized photooxygenation of p-hydroxyphenylpyruvic acid (1) to a quinol intermediate which, on raising the pH is converted to homogentesic acid (2) has been studied in detail as a possible model for the biological conversion of 1 to 2. It was found, however, that the mechanism of the enzymic conversion of 1 to 2 differs from that of the photooxygenation.

PHS-6040
(12-75)

Project Description:

The previously reported dye-sensitized photooxygenation of p-hydroxyphenylpyruvic acid (1) to a p-quinol (3) which is then converted at high pH to homogentesic acid (2) has been studied in greater detail. The p-quinol 3 cyclizes reversibly to form a bicyclic compound. Dye-sensitized photooxygenation provides an efficient and simple method for the synthesis of p-quinols and their cyclyzed products. However, the p-quinol 3 was subsequently found not to be a substrate for p-hydroxyphenylpyruvate dioxygenase which catalyzes the conversion of 1 to 2 (Nakai et al., Biochem. Biophys. Res. Commun. 67: 590, 1975).

Publications:

1. Saito, I., Chujo, Y., Shimazu, H., Yamane, M., Matsuura, T. and Cahnmann, H. J.: Nonenzymic oxidation of p-hydroxyphenylpyruvic acid with singlet oxygen to homogentisic acid. A model for the action of p-hydroxyphenylpyruvate hydroxylase. J. Am. Chem. Soc. 97: 5272-5277, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45003-02 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Model Reactions for the Synthesis of Thyroxine Residues in Thyroglobulin

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

PI:	J. Pommier	Visiting Associate	CEB NIAMDD
	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD
OTHER:	J. Nunez	Director of Research	C.N.R.S.

COOPERATING UNITS (if any)

Unité de Recherche sur la Glande Thyroïde et la Régulation Hormonale, INSERM, Bicêtre, France

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

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

Enzymatic iodination was compared with chemical iodination of various copolymers and oligopeptides of Tyr. Iodination catalyzed by lactoperoxidase (LPO) or thyroid peroxidase (TPO) was always more efficient than iodination catalyzed by horse radish peroxidase or nonenzymic iodination. A linear random copolymer of Lys and Tyr gave, with TPO or LPO, thyroxine (T4) in excellent yield. The kinetics of T_4 formation with TPO was studied.

Project Description:

The linear random copolymer of L-Lys and L-Tyr described in last year's report is a model for thyroglobulin since thyroid peroxidase (TPO), in the presence of iodide and a hydrogen peroxide generating system, converts the Tyr residues to mono- and diiodotyrosine (MIT and DIT) residues and furthermore converts some of the DIT residues to thyroxine (T_4) residues.

TPO can be replaced with lactoperoxidase (LPO). The T_4 yield was 14-15% with TPO and only slightly less with LPO. A kinetic study, using TPO, showed that all Tyr residues must be iodinated and nearly all MIT residues converted to DIT residues before T_4 formation begins. MIT residues did not react with DIT residues to form triiodothyronine.

Among various copolymers tested in addition to the Lys-Tyr copolymer, only one, a linear random copolymer of L-Glu and L-Tyr, gave T_4, yet only in low yield. Although nome of the other copolymers (linear, multichain, ordered and random) yielded T_4, they could be iodinated enzymically or chemically (I_2, pH 9) to some extent. When horse radish peroxidase was used as the iodinating enzyme the degree of iodination was considerably less with all polymers.

A number of di- and tripeptides of Tyr were tested with TPO. All of them were more or less iodinated with the formation of MIT and DIT residues, but none of them yielded a T_4 peptide.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45004-05 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structure of Polypeptide and Protein Hormones and Lipoproteins

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

PI:	H. Edelhoch	Senior Scientist	CEB NIAMDD
	K. Ingham	Staff Fellow	CEB NIAMDD
	G. Palumbo	Guest Worker	CEB NIAMDD
	J. Osborne	Staff Fellow	CEB NIAMDD
	S. Formisano	Guest Worker	CEB NIAMDD
OTHER:	R. E. Lippoldt	Sr. Health Services Officer	CEB NIAMDD
	B. Brewer	Branch Chief	MDB NHLI

COOPERATING UNITS (if any)

National Heart and Lung Institute

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.8	3.6	.2

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

The interactions of high density lipoproteins, apoA-I and apoA-II, with the phospholipid, lysolecithin, have been evaluated in order to obtain the affinity constants and structural changes in the proteins produced by the phospholipid.

The rates of combination of the two subunits of human chorionic gonadotropin have been evaluated. The data are best described by a second order recombination and a first order conformational transition.

The role of water in stabilizing the properties of proteins, nucleic acids and membranes has been reviewed.

PHS-6040
(12-75)

Project Description:

A comprehensive thermodynamic analysis has been made of the forces responsible for the stability of the major classes of biological structures which are organized by noncovalent interactions, i.e., proteins, nucleic acids and membranes. It has been shown that the folding of these structures can be understood in terms of the thermodynamics of the interactions between the non-polar moieties of the proteins and membranes, i.e., surfactants with water or between the bases of the nucleic acids with water. The most characteristic property of these two systems is the heat capacity. We have calculated the heat capacity change as a function of temperature from the critical micelle concentrations of numerous surfactant molecules. The changes for the nucleic acids were obtained from calorimetric data. The fundamental role played by water in stabilizing these structures has been evaluated.

A study of the mechanism by which the peroxidase of the thyroid gland converts iodide into MIT and DIT and then T_3 and T_4 in the protein, i.e., thyroglobulin, has been initiated in Paris in the laboratory of Dr. J. Nunez. Since the thyroid peroxidase is still difficult to purify in quantity, we have used lactoperoxidase for the initial studies. We have evaluated 4 independent reactions of the peroxidase in order to understand its properties.

1) catalatic - decomposition of peroxide
2) iodine formation
3) iodination of tyrosyl compounds
4) oxidation of tyrosyl compounds

The effects of different goiterogens on these reactions are being evaluated.

The properties of the α and β subunits of human chorionic gonadotropin have been evaluated from the fluorescence behavior of the tyrosyl chromophores. It has also been shown that the two subunits recombine by a bimolecular reaction to give an intermediate which then undergoes a conformational change to give the native hormone. The kinetics of recombination have been measured over a 100-fold change in concentration. The data have been analyzed by several mechanisms and shown to conform closest to the above description.

Publications:

1. Edelhoch, H. and Osborne, J. C., Jr.: The Thermodynamic Basis of the Stability of Proteins, Nucleic Acids and Membranes. In Anfinsen, C.B., Edsall, J.T. and Richards, F.M. (Eds.): Advances in Protein Chemistry. New York, Academic Press, 1976, Vol. 30, pp. 183-250.

2. Gwynne, J., Palumbo, G., Brewer, H. B., Jr. and Edelhoch, H.: The interaction of ApoA-I from human high density lipoprotein with lysolecithin. J. Biol. Chem. 250: 7300-7306, 1975.

3. Gwynne, J., Palumbo, G., Osborne, J. C., Jr., Brewer, H. B., Jr. and Edelhoch, H.: The self-association of ApoA-II, an apoprotein of the human high density lipoprotein complex. Arch. Biochem. Biophys. 170: 204-212, 1975.

4. Ingham, K. C., Saroff, H. A. and Edelhoch, H.: Ligand-induced self-association of human luteinizing hormone. Negative cooperativity in the binding of 8-anilino-1-naphthalenesulfonate. Biochemistry 14: 4745-4751, 1975.

5. Ingham, K. C., Saroff, H. A. and Edelhoch, H.: Ligand-induced self-association of human chorionic gonadotropin. Positive cooperativity in the binding of 8-anilino-1-naphthalenesulfonate. Biochemistry 14: 4751-4758, 1975.

6. Osborne, J. C., Jr., Palumbo, G., Brewer, H. B., Jr. and Edelhoch, H.: The self-association of the reduced ApoA-II apoprotein from the human high density lipoprotein complex. Biochemistry 14: 3741-3746, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45005-13 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Measurement of Iodo Compounds in Biological Materials

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

PI:	C. G. Lewallen	Medical Director	CEB NIAMDD
OTHER:	H. Hoch	Research Scientist	VA Hospital

COOPERATING UNITS (if any)
Veterans Administration Center, Martinsburg, West Virginia

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

Terminated

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45006-08 CEB

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Thyroid Iodoproteins

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

PI:	A. E. Burkhardt	Staff Fellow	CEB	NIAMDD
	J. E. Rall	Director, Intramural Programs	ODIRP	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	.2

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

The incorporation of ^{3}H-leucine into guinea pig thyroglobulin was studied in vivo. The distribution of ^{3}H-leucine among the three bands of reduced guinea pig thyroglobulin was determined for both methimazole treated animals as well as control (non-treated) animals to determine what effect iodination has on the subunit composition of thyroglobulin.

PHS-6040
(12-75)

Project Description:

The goal of this project is to determine the nature and origins of the primary polypeptide chains of the major thyroid iodoprotein, 19S thyroglobulin (MW 660,000).

Previous work had shown that guinea pig 19S thyroglobulin, after reduction and alkylation, possessed three main bands (called A, B and C) when analyzed by SDS gel electrophoresis. The molecular weights of the bands had been shown to be 295,000 (A band); 210,000 (B band); and 110,000 (C band). Studies of the relative amounts of the three bands in guinea pig thyroglobulin of varying iodine content showed that in low iodine thyroglobulin the heaviest species (the A band) predominated while in more heavily iodinated thyroglobulin the smallest species (the C band) was the major component. These results implied that (1) iodination may play a role in converting the A band into B and C bands and (2) only the largest band, A, is synthesized by the cell.

The present work was designed to test these hypotheses by the use of *in vivo* ^{3}H-leucine incorporation studies. The procedure used was to inject guinea pigs i.p. with ^{3}H-leucine and then sacrifice the animals at intervals ranging from 1 hour to 96 hours after the injection. The thyroglobulin from these animals was purified and analyzed, after reduction, by SDS gel electrophoresis to determine the ^{3}H-leucine content of the three bands. One group of guinea pigs was treated with methimazole to block iodination of the newly synthesized thyroglobulin while a second set, not treated with the drug, served as controls.

The results of the study showed that ^{3}H-leucine appeared largely in the A band at early times, but with longer time intervals, appeared in increasing amounts in the smaller B and C bands. At 5 hours after the injection of ^{3}H-leucine in the control animals, the following relative distribution of ^{3}H-leucine was observed (the numbers are the dpm/microgram of protein normalized so that the value for the C band equals 1.0).

A band:	21.0
B band:	2.9
C band:	1.0

By 96 hours aftter the injection, the distribution had changed to:

A band:	5.1
B band:	2.2
C band:	1.0

These results support the hypothesis that the A band (MW 295,000) is the polypeptide chain synthesized by the cell and that the smaller B and C bands derive from the A band.

Treatment with methimazole, however, did not alter the relative incorporation of ^{3}H-leucine into the three bands. After 5 hours the relative distribution of ^{3}H-leucine in the methimazole treated animals was:

A band:	25.8
B band:	3.0
C band:	1.0

At 96 hours the distribution was:

A band:	5.2
B band:	2.2
C band:	1.0

Separate control experiments showed that the methimazole treatment used reduced ^{125}I uptake in the treated animals to 1-8% of that in the control animals. The appearance of the smaller B and C bands in the methimazole treated animals with roughly the same rate as in the control animals suggests that idoination *per se* does not produce the B and C bands from the A band.

The slow rate of incorporation of ^{3}H-leucine into B anc C (at 96 hours, the incorporation into B and C was still increasing) suggests that B and C are not synthesized within the cell as separate chains. Also control experiments performed in this study showed that the conversion of the A band into B and C did not occur *in vitro* during the purification of thyroglobulin from the thyroids, but rather the conversion of A into the smaller bands was the result of an *in vivo* process.

Publications:

1. Haeberli, A., Bilstad, J., Edelhoch, H. and Rall, J. E.: The elementary chain composition of guinea pig thyroglobulin. J. Biol. Chem. 250: 7294-7299, 1975.

2. Haeberli, A., Salvatore, G., Edelhoch, H. and Rall, J. E.: Relationship between iodination and the polypeptide chain composition of thyroglobulin. J. Biol. Chem. 250: 7836-7841, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 ZM 45007-11 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Protein Synthesis in the Thyroid Gland

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

PI:	M. Izumi	Visiting Associate	CEB NIAMDD
	J. Robbins	Chief, Clin. Endo. Br.	CEB NIAMDD
	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.4	.3	.1

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

Work has continued on the isolation and characterization of abnormal iodoproteins present in the soluble fraction of a transplantable rat thyroid tumor (Wollman Line 1-8R). Since these proteins react with antiserum against normal thyroglobulin (Tg), they appear to be abnormal thyroglobulins synthesized by the tumor. Affinity chromatography employing anti-Tg immunoglobulin fixed to agarose (see last year's report), as well as conventional separation method, were used to partially purify two components. The major fraction ("Peak A") was excluded from gels of high pore size; and had a low sedimentation rate (∿8S), but did not contain aggregates. Equilibrium sedimentation in CsCl gradients showed it to have an unusually high density (∿1.4). Since treatment with crude glycosidase lowered the density to ∿1.3, this protein appears to have a very high content of carbohydrate. A second fraction ("Peak B") was retarded by gel filtration and therefore was a more symmetrical molecule; it also had a more "normal" density of ∿1.3. Since neither protein contained thyroxine or triiodothyronine after prolonged in vivo labeling with ^{125}I, it is possible that their abnormal structure prevented iodotyrosine coupling.

PHS-6040
(12-75)

Project No. Z01 AM 45007-11 CEB

Project Description:

Work has continued on the isolation and characterization of abnormal iodoproteins present in the soluble fraction of a transplantable rat thyroid tumor (Wollman Line 1-8R). Since these proteins react with antiserum against normal thyroglobulin (Tg), they appear to be abnormal thyroglobulins synthesized by the tumor. Affinity chromatography employing anti-Tg immunoglobulin fixed to agarose (see last year's report), as well as conventional separation method, were used to partially purify two components. The major fraction ("Peak A") was excluded from gels of high pore size; and had a low sedimentation rate (∿8S), but did not contain aggregates. Equilibrium sedimentation in CsCl gradients showed it to have an unusually high density (∿1.4). Since treatment with crude glycosidase lowered the density to ∿1.3, this protein appears to have a very high content of carbohydrate. A second fraction ("Peak B") was retarded by gel filtration and therefore was a more symmetrical molecule; it also had a more "normal" density of ∿1.3. Since neither protein contained thyroxine or triiodothyronine after prolonged *in vivo* labeling with ^{125}I, it is possible that their abnormal structure prevented iodotyrosine coupling.

Publications:

1. Izumi, M., Cahnmann, H.J., Robbins, J.: Isolation by affinity chromatography of rat thyroid tumor protein related to thyroglobulin. In Robbins, J. and Braverman, L.E. (Eds.): Thyroid Research. Excerpta Medica, Amsterdam, 1976, p. 555.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 45008-03 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Phosphoprotein Phosphatase of the Thyroid Gland

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

PI:	M. McClung	Clinical Associate	CEB	NIAMDD
	J. E. Rall	Director, Intramural Research Programs	ODIRP	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.6	0

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

We were successful in characterizing and partially purifying phosphoprotein phosphatase from bovine thyroid glands. The effects that divalent cations, salts, cAMP, cGMP, and ATP have on modulation of the reaction rate were studied.

Studies on the characterization and purification of phosphoprotein phosphatase in normal placental tissue is in progress.

PHS-6040
(12-75)

Project No. Z01 AM 45008-03 CEB

Project Description:

It is now apparent that many of the actions of TSH in the thyroid involve the adenyl cyclase-cAMP pathway. Following Greengard's proposal that cAMP effects in many tissues are mediated by activation of cAMP-dependent protein kinases, several laboratories have focused their attention upon the identification and characterization of these enzymes in thyroid glands. While phosphorylation of specific protein substrates, as yet to be identified, has been suggested as an important step in the mechanism of action of TSH, only the phosphorylation reaction has been well studied. In order for protein phosphorylation to be an effective regulatory mechanism, an active dephosphorylating system must also be present.

We were successful in characterizing a phosphoprotein phosphatase from bovine thyroid glands and purified this phosphatase 20-fold. Upon DEAE-cellulose chromatography, a single peak of activity is found which is capable of utilizing both phosphoprotamine and phosphohistone as substrate. The apparent K_m for protamine dephosphorylation is 0.7 μM while that for histone is 11.9 μM. Optimal pH is in the neutral range. Although not required for activity, divalent cations can modulate the reaction rate. Co^{++} and Mn^{++} enhance activity; Zn^{++}, Fe^{++}, and Ca^{++} are inhibitory; and Mg^{++} does not change the activity. The addition of cAMP, cGMP, or ATP has no effect, while NaF, potassium phosphate, pyrophosphate, and p-nitrophenyl phosphate each markedly inhibits activity.

We also extended our interests of the dephosphorylating system to the placenta where we were able to characterize and partially purify phosphoprotein phosphatase in normal placental tissue. Further work on the characterization of the placental phosphatase is in progress.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45009-09 CEB

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies in Thyroid Disease

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

PI:	J. Robbins	Chief, Clin. Endo. Br.	CEB NIAMDD
	B. D. Weintraub	Senior Surgeon	CEB NIAMDD
	M. C. Gershengorn	Clinical Associate	CEB NIAMDD
OTHER:	M. McClung	Clinical Associate	CEB NIAMDD
	M. A. Beaven	Research Pharmacologist	PB NHLI
	A. E. Broadus	Clinical Associate	EB NHLI
	E. W. Chu	Head, Cytopathology Section	LP NCT
	P.R. Larsen	Head, Thyroid Unit	Peter Bent Brigham Hospital

COOPERATING UNITS (if any)

National Heart and Lung Institute, National Cancer Institute, Peter Bent Brigham Hospital

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	.8	.2

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

Thyroid cancer diagnosis: Large oral doses of TRH (200 mg per day) were used as a method for evaluating the function of metastic thyroid cancer. TRH had a persistent stimulatory effect on TSH secretion for 14 days. Stimulation of radioiodine uptake in the tumor was more effective with oral TRH than with the customary injection of bovine TSH. Further study is in progress to compare the uptake after TRH with that after endogenous hypothyroidism alone.

Medullary thyroid carcinoma contains a high histaminase level. Using the ^{3}H release method of Beaven and Jacobsen, we found that tumor aspirate (3-5 µl) had histaminase activity 30 fold higher than normal or in other types of thyroid nodules. The cytology also was diagnostic of medullary carcinoma. Therefore, fine needle aspiration is useful in the diagnosis of this type of thyroid cancer.

Hyperthyroidism: Most cases of hyperthyroidism result from a primary defect, or an autoimmune process involving the thyroid gland. We have studied an 18 year old hyperthyroid woman with persistently elevated serum TSH and no evidence of pituitary tumor during 11 years of observation. This patient represents a new syndrome of TSH-induced hyperthyroidism, apparently caused by a selective partial resistance of the pituitary thyrotrophs to the action of thyroid hormone.

Project Description:

Thyroid cancer diagnosis: Thyrotropin releasing hormone (TRH), the hypothalamic hormone which stimulates pituitary synthesis and secretion of thyroid stimulating hormone (TSH) is used in a small intravenous dose (500 µg or less) for clinical testing of pituitary and thyroid disease. We have investigated the utility of larger oral doses of TRH (200 mg per day) as a method for evaluating the presence and the functional capacity of metastatic thyroid cancer. In 9 athyreotic patients with thyroid cancer, TRH had a persistent stimulatory effect on TSH secretion for 14 days. Stimulation of radioiodine uptake in the tumor was more effective with oral TRH than with the customary injection of bovine TSH. Further study is in progress to compare the uptake after TRH with that after endogenous hypothyroidism without TRH.

The use of fine needle aspiration biopsy is being investigated as part of a study of methods to improve preoperative diagnosis of thyroid nodules. The accuracy of the cytologic diagnoses is now being evaluated. In the course of this study, we investigated the utility of histaminase measurement in the diagnosis of medullary thyroid carcinoma, since this tumor is known to contain a high histaminase level. Using the ^{3}H release method of Beaven and Jacobsen, we found that the tumor aspirate (3-5 µl) had histaminase activity which was 30 fold higher than in normal thyroid or in other types of thyroid nodules. The cytology and Congo-red staining of this tumor also was diagnostic of medullary carcinoma. Therefore, fine needle aspiration is useful in the diagnosis of this type of thyroid cancer.

Hyperthyroidism: Although most cases of hyperthyroidism result from a primary defect in the thyroid gland itself, or an autoimmune process involving the thyroid, a few cases secondary to hypersecretion of pituitary TSH have recently been reported, usually with a coexisting pituitary tumor. We have studied an 18 year old hyperthyroid woman with persistently elevated serum TSH and no evidence of pituitary tumor during 11 years of observation. TRH produced an increase of serum TSH followed by an increase of serum T_3, indicating that the secreted TSH was biologically active. Exogenous T_3 produced a decrease in TSH and in TRH response, but at inappropriately high serum T_3 levels. This patient represents a new syndrome of TSH-induced hyperthyroidism, apparently caused by a selective, partial resistance of the pituitary thyrotrophs to the action of thyroid hormone.

Role of T_4 in pituitary-thyroid feedback axis: The changes in serum triiodothyronine (T_3), thyroxine (T_4), and thyrotropin (TSH) were measured during iodine repletion in a woman who was severely iodine-deficient because of a congenital iodide-trapping defect. Serum T_3 became detectable 12 hours after iodine was begun and reached 68 ng/dl, a level within the normal range, by 36 hours. It rose progressively during the first 9 days reaching a supra-normal level and then fell slowly to 130 ng/dl. Serum T_4 was not detected

until the 9th day and then rose to 6.8 µg/dl. Serum TSH fell rapidly during the first 9 days (disappearance rate was 0.17/d); there was a significant negative correlation with serum T_3. From the 10th through the 32nd day serum TSH fell more slowly (disappearance rate was 0.05/day) and correlation was with serum T_4 not T_3. It appears that both T_3 and T_4 directly regulate pituitary TSH secretion.

Publications:

1. Gershengorn, M. C. and Weintraub, B. D.: Thyrotropin-induced hyperthyroidism caused by selective pituitary resistance to thyroid hormone. J. Clin. Invest. 56: 633-642, 1975.

2. Gershengorn, M. C., Weintraub, B. D., and Robbins, J.: Response to oral thyrotropin releasing hormone in athyreotic patients with thyroid carcinoma. In Robbins, J. and Braverman, L. E. (Eds.): Thyroid Research. Excerpta Medica, Amsterdam, 1976, p. 575.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45010-06 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Ectopic Placental Protein and Subunit Production by Tumors

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

PI:	S. W. Rosen	Medical Director	CEB NIAMDD
	B. D. Weintraub	Sr. Surgeon	CEB NIAMDD
OTHER:	H. Sussman	Assistant Prof. Med.	Stanford Univ. Med. Ctr.
	R. Kahn	Sr. Surgeon	DB NIAMDD
	P. Gorden	Clinical Director	NIAMDD
	H. Brereton	Head, Rad. Med. Sec.	RB NCI
	J. Lieblich	Clinical Associate	CEB NIAMDD
	A. Rabson	Director, DCBD	OD NCI
	C. Kim	Postdoctoral	LP NCI
	P. Kohler	Assoc. Prof. Medicine	Baylor
	W. Sindelar	Clinical Associate	DCT NCI
	J. Chou	Staff Fellow	LBS NICHD
	J. Robinson	Branch Chief	LBS NICHD
	E. Maxwell	Research Chemist	LMB NIAMDD
	G. Cox	Staff Fellow	LMB NIAMDD

COOPERATING UNITS (if any)
Stanford University Medical Center, Palo Alto, CA; National Cancer Institute; Baylor College of Medicine, Houston, TX

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	2.0	1.2

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

We are studying the incidence and specificity of placental proteins (chorionic gonadotropin and its free alpha and beta subunit, placental lactogen, and placental alkaline phosphatase) as cancer markers. These proteins appear to distinguish cancers from benign tumors and correlate with the response to therapy. Various permanent cell lines have been established to study the regulation of placental protein synthesis in vitro and to characterize the properties of these materials. We have succeeded in translating placental messenger RNA in a cell-free wheat germ system and in characterizing the various products by immunologic, physicochemical, and biologic methods.

PHS-6040
(12-75)

Project Description:

A. In Vivo Studies

We have continued to study the incidence and specificity of neoplastic placental protein production : chorionic gonadotropin (HCG), HCG-β subunit, HCG-α subunit, placental alkaline phosphatase (PAP), and placental lactogen (HPL). Our previous studies had indicated that the combined incidence of these markers was about 6% in lung cancer. We have now found a roughly comparable incidence in breast cancer and pancreatic carcinoma. These incidence figures are in striking contrast to those observed in islet cell tumors of the pancreas. In a large retrospective series the incidence of ectopic placental peptides was about 50% in functional islet cell carcinomas but 0% in islet cell adenomas. Ectopic peptides were found associated with overproduction of insulin, gastrin, and the factor producing the "watery diarrhea" syndrome. High concentrations of these proteins were measured in extracts of certain islet cell cancers and the serum concentrations correlated well with response to chemotherapy. These ectopic proteins may be a valuable marker for islet cell cancers and may aid in the otherwise difficult differentiation from benign islet cell tumors.

B. Characterization of Placental Proteins Secreted by Various Human Cancer Cell Lines

We have previously reported detailed studies of HCG, HCG-α, HCG-β, and PAP production by clonal strains derived from a bronchogenic carcinoma and choriocarcinoma. Recently we have discovered that various other human cancer cell lines secrete these proteins. Of particular importance is the fact that HeLa cells (the oldest permanent human cell lines, originally derived from cancer of the cervix) secrete large amounts of alpha. This is the first extracellular product of HeLa to be characterized and its identification will be of general interest to cell biologists using this line to study basic regulatory mechanisms in protein biosynthesis. We are currently studying the effects of cyclic nucleotides, growth factors, butyrate, hypothalamic releasing hormones, and other agents on the regulation of ectopic protein production.

Three of the ectopic alphas identified to date have apparently higher molecular weights than normal subunits in gel chromatography. Two such forms have been extensively purified and have been shown to differ from normal alpha in beta-combining properties, mobility on SDS gels, and amino acid composition. Studies are in progress to determine whether these abnormal forms are the true precursors of normal alpha.

C. Cell Free Translation of Placental Protein Messenger RNA

We have succeeded in translating messenger RNA from normal term human placentas using wheat germ cell-free extracts. Under a variety of conditions two products of this translation have been identified. 1) A 19,000-20,000 molecular weight form with immunologic, physical, and chemical (i.e. tryptic

peptides) characteristics identical to authentic HPL. 2) A 22,000-24,000 molecular weight form with similar immunologic and chemical properties but with different physical properties. Currently studies are in progress to define the lactogenic receptor-binding properties of each of these two forms and to determine whether the larger form is a physiologic precursor of the smaller form.

Using similar methods we are also attempting to translate messenger RNA from HeLa, ChaGo and other cell lines which make glycoprotein hormones and their subunits. These studies should allow us to determine whether there are independent messenger RNA's for alpha and beta subunits and whether there are precursor subunit forms, as had been suggested by our previous studies with tumor subunits.

Publications:

1. Weintraub, B. D., Krauth, G., Rosen, S. W. and Rabson, A. S.: Differences between purified ectopic and normal alpha subunits of human glycoprotein hormones. J. Clin. Invest. 56: 1043-1053, 1975.

2. Lieblich, J. M., Weintraub, B. D., Rosen, S. W., Chou, J. Y. and Robinson, J. C.: HeLa cells secrete α subunit of glycoprotein tropic hormones. Nature 260: 530-532, 1976.

3. Cox, G. S., Weintraub, B. D., Rosen, S. W. and Maxwell, E. S.: Properties of biologically active messenger RNA from human placenta. J. Biol. Chem. 251: 1723-1730, 1976.

4. Muggia, R. M., Rosen, S. W., Weintraub, B. D. and Hansen, H. H.: Ectopic placental proteins in nontrophoblastic tumors. Cancer 36: 1327-1337, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45012-03 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Growth Hormone Heterogeneity

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

PI:	S. W. Rosen	Medical Director	CEB NIAMDD
OTHER:	R. C. Dimond	Research Scientist	Walter Reed Army Institute

COOPERATING UNITS (if any)

Walter Reed Army Institute of Research

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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

Inactive

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45013-04 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

TSH and Prolactin Studies; Subunits of Glycoprotein Hormones

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

PI:	B. D. Weintraub	Senior Surgeon	CEB NIAMDD
	S.W. Rosen	Medical Director	CEB NIAMDD
OTHER:	M. Blackman	Postdoctoral Fellow	CEB NIAMDD
	M. Gershengorn	Clinical Associate	CEB NIAMDD
	I. Kourides	Instructor in Medicine	Mass. General Hospital
	F. Maloof	Chief, Thyroid Unit	Mass. General Hospital
	A. Rogol	Assistant Professor	Duke University

COOPERATING UNITS (if any)

Harvard Medical School, Boston, Massachusetts; Duke University, Durham, North Carolina

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endorine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.1	1.9	1.2

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

Glycoprotein hormones and free subunits are secreted by animal and human pituitaries in vivo and in vitro. Pituitary tumors may secrete alpha subunits and TSH in an abnormal fashion and are autonomous to normal regulatory factors. Studies are underway to define the factors controlling subunit synthesis, combination, and secretion in normal and neoplastic pituitaries. TSH and prolactin secretion appear to be independently regulated.

PHS-6040
(12-75)

Project Description:

A. Measurement of Glycoprotein Hormone Subunits in Human Serum, Cerebrospinal Fluid, and Pituitary Gland.

Previous studies from our laboratory have established that free alpha and beta subunits of glycoprotein hormones may be secreted from the pituitary in normal human subjects; the rate of this subunit secretion is increased in primary hypothyroidism (α and TSH-β), primary hypogonadism (α and LH-β), after thyrotropin releasing hormone (α and TSH-β) and luteinizing hormone releasing hormone (α and LH-β). We have recently demonstrated that certain pituitary tumors (5 of 60) may secrete even larger amounts of alpha subunit than that described for the non-neoplastic pituitary. Such neoplastic alpha secretion is not accompanied by any free beta subunits and is unresponsive to either of the hypothalamic releasing hormones. Of the 2 tumors with suprasellar extension both showed markedly identical cerebrospinal alpha concentrations. After surgical removal of the tumors serum and CSF alpha levels fell to the normal range and tumor extracts showed high alpha concentrations. Since none of 25 cases of proven "empty sella" syndrome was associated with increased subunits, elevated and autonomous alpha production may identify the presence of a tumor in certain patients with an enlarged sella turcica.

B. Regulation of Biosynthesis and Secretion of TSH and Subunits by Pituitary Explants and Dispersed Cell Cultures.

In addition to our previously published method for measurement of rat TSH, we have recently developed radioimmunoassays for rat and mouse alpha and TSH-β. Using these assays we have demonstrated that normal rat pituitary explants and dispersed cell cultures secrete large amounts of free alpha subunit but much smaller amounts of TSH-β. The secretion of subunits as well as of TSH is highly responsive to TRH. In contrast, certain mouse thyrotropic tumors (Furth) which we have maintained by serial transplantation, secrete a large excess of free alpha subunits and appear less responsive to TRH. These two *in vitro* models of normal and neoplastic pituitary function are currently being used to elucidate the direct roles of TRH, somatostatin, calcium, glucocorticoids, dopamine, thyroid hormone, etc. on the biosynthesis and secretion of TSH and subunits.

C. Combination of Subunits of Glycoprotein Hormones.

Using newly developed micromethods, we have studied the detailed kinetics of subunit combination *in vitro* as measured by receptor assay (RRA) and immunoassay (RIA). Various preparations of subunits differ greatly in the amount physically combined (as defined by RIA and gel chromatography) as well as in the proportion of these combinations which have receptor-binding activity. Furthermore, kinetic studies reveal that physical combination preceeds the appearance of receptor activity, providing the first direct evidence for a previously postulated "intermediate" in alpha-beta combination.

D. Inappropriate Secretion of TSH.

We have studied additional cases (total of 8) of inappropriately high TSH secretion in the presence of elevated concentrations of total and free thyroid hormones. The syndromes are heterogeneous in origin and in their natural history: 4 are secondary to a pituitary chromophobe adenoma, 3 are related to selective pituitary resistance to the action of thyroid hormone, and 1 to generalized resistance to thyroid hormone. We have found that the TSH secondary to neoplasms is generally unresponsive to TRH stimulation and thyroid hormone suppression, while TSH secondary to thyroid hormone resistance can be stimulated or suppressed, albeit in a quantitatively abnormal fashion. Furthermore we have found very high circulating alpha in 3 of the 4 tumor cases but in none of the 4 non-neoplastic cases.

E. Abnormal Forms of Subunits

We have continued to characterize several apparently large forms of alpha subunit, HCG-β and TSH-β secreted by normal pituitary as well as pituitary and other tumors in vivo and in vitro. These forms have diminished or absent ability to combine with the opposite subunit and may represent true precursor forms. Studies are in progress to directly demonstrate the precursor nature of these forms by pulse labeling - chase techniques with labeled amino acid precursors.

F. Metabolic Clearance (MCR) and Production Rates (PR) of Glycoprotein Hormones Subunits.

Studies continue on the MCR and PR of subunits in man. We have previously shown very high MCR of HCG-α, TSH-α and TSH-β compared to complete TSH or HCG. Subunit MCR are related to body surface area, thyroid and renal function. There is normally a very high basal PR of alpha (consistent with our previously described large alpha pool in the pituitary) while secretion of TSH-β is low. In primary hypothyroidism the increased biosynthesis of alpha and TSH-β is efficiently combined into complete TSH and there is little increase in free subunit production rates.

G. Human Prolactin Studies

We continue to study the relationship of prolactin and TSH secretion in man. It is now clear that various doses and duration of glucocorticoid administration can blunt TSH production directly but have no effect on prolactin production rates. This differential effect has now been extended to the 4 cases of inappropriate secretion of TSH caused by target organ resistance to thyroid hormone. In these cases, also, glucocorticoids can produce marked decreases in TSH production without affecting prolactin production. These data support our previous studies of independent physiologic regulation of TSH and prolactin, despite the fact that both are stimulated by exogenous TRH.

Publications:

1. Gershengorn, M. C. and Weintraub, B. D.: Thyrotropin-induced hyperthyroidism caused by selective pituitary resistance to thyroid hormone. J. Clin. Invest. 56: 633-642, 1975.

2. Kourides, I. A., Weintraub, B. D., Rosen, S. W., Ridgway, E. C., Kilman, B. and Maloof, F.: Secretion of alpha subunit of glycoprotein hormones by pituitary adenomas. J. Clin. Endo. Metab. June, 1976 (in press).

3. Ingham, K. C., Weintraub, B. D. and Edelhoch, H. Kinetics of recombination of the subunits of human chorionic gonadotropin. Effect of subunit concentration. Biochemistry 15: 1720-1726, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45014-05 CEB

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Adrenal Secretion

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

PI: J. Wolff Medical Director CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.4	.2

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

The locus and nature of the choleragen and endotoxic lipopolysaccharide effects on steroid secretion by adrenal cells in culture has been studied by the use of mutant cell lines, receptor competition and the use of gangliosides. The receptor for ACTH has a different locus than those for these toxins. In addition the role of adenosine-containing compounds in steroidogenesis and adenylate cyclase activation has been elucidated. For all stimulators the effect appears to be on the external surface of the cell membrane via activation of adenylate cyclase.

PHS-6040
(12-75)

Project No. Z01 AM 45014-05 CEB

Project Description:

Studies on adrenal and Leydig cell tumor cells in culture have dealt with two aspects: the effect of bacterial toxins and the effect of adenosine.

Choleragen stimulates steroid secretion and adenylate cyclase in three cell lines, adrenal tumor line (Y-1), a corticotropin-resistant mutant derived from Y-1 called OS-3, and a receptor-deficient Leydig tumor line (I-10). Sensitivity for half-maximal stimulation varies from 3 to 36 pM choleragen, the I-10 line being the most sensitive. Latency before the onset of steroidogenesis is longer in OS-3 and I-10 cells than in the Y-1 line. In both OS-3 and I-10 cells choleragen stimulates adenylate cyclase whether ITP or 5'-guanylylimidodiphosphate is the regulatory cofactor used. In addition to the responses of the receptor-deficient lines, choleragen does not, during its latency, block the response to corticotropin in Y-1 cells; corticotropin does not block binding of ^{125}I-labeled choleragen to Y-1 cells; gangliosides do not interfere with the corticotropin-induced stimulation of Y-1 cells. We conclude that the corticotropin and choleragen receptors are different.

Lipopolysaccharides (endotoxins) from Escherichia coli, Serratia marcescens and Salmonella typhosa stimulated steroid production in Y-1 adrenal tumor cells in culture with a latent period of 3-4 h. Lipid A, derived from Escherichia coli lipopolysaccharide, also stimulated steroidogenesis. Lipopolysaccharides and lipid A also stimulate adenylate cyclase activity and cause rounding of the cells. In contrast, lipopolysaccharides do not stimulate as judged by the failure of lipopolysaccharides to block, during their latency, the response to corticotropin in Y-1 cells.

Studies with adenosine have shown that steroidogenesis by Y-1 adrenal tumor cells in culture is stimulated by ATP, App(NH)p, APP(CH_2)p, ADP, AMP, NAD, FAD and adenosine but not by adenine or other nucleoside triphosphates. ATP and adenosine are active in the micromolar range. Like ACTH the onset of stimulation is immediate and occurs to the same extent. 2'- and 5'-deoxyadenosine and 2-chloroadenosine are also active. Stimulation is accompanied by rounding of the cells. Dipyridamol, an inhibitor of adenosine transport, increased the response to low concentrations of adenosine, suggesting that adenosine acts externally.

Stimulation of steroidogenesis by phosphorylated adenosine compounds fails to occur in the presence of adenosine deaminase, and a requirement for the conversion of the above compounds to adenosine seems probable. The inhibition of cAMP effects by adenosine deaminase suggests that some of its effects are also indicated by conversion to adenosine. Similar stimulation is seen in I-10 Leydig tumor cells, but an ACTH-resistant mutant of Y-1 cells, called OS-3, is relatively resistant to adenosine.

Adenosine and 2-chloroadenosine stimulate adenylate cyclase in membranes from Y-1 and I-10 cells at concentrations slightly greater than are effective for steroidogenesis. Other nucleosides are ineffective. Like $^{1-24}$ACTH, the adenosine effect in Y-1 membranes is rapid and is on the V_{max} intercept (vs ATP) and not on the K_m. In contrast to steroidogenesis, adenosine is only a partial agonist for adenylate cyclase. Its effect occurs in the presence of ITP, GTP or Gpp(NH)p.

Theophylline inhibits steroidogenesis competitively, and simultaneously blocks the rounding response at concentrations when there is little effect on phosphodiesterase. Inhibition of adenylate cyclase occurs in the same concentration range but is of the mixed type.

Publications:

1. Wolff, J. and Cook, G.H.: Choleragen stimulates steroidogenesis and adenylate cyclase in cells lacking functional hormone receptors. Biochim. Biophys. Acta 413: 283-290, 1975.

2. Wolff, J. and Cook, G.H.: Endotoxic lipopolysaccharides stimulate steroidogenesis and adenylate cyclase in adrenal tumor cells. Biochim. Biophys. Acta 413: 291-297, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45015-06 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Thyroid Plasma Membranes

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

PI:	J. Wolff	Medical Director	CEB NIAMDD
	R. F. Asbury	Clinical Associate	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.3	.4

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

Polycations have been shown to activate adenylate cyclase in beef thyroid membranes, intact adrenal and Leydig tumor cells, and in membranes derived from these tissue culture cells. The effect is due to charge-charge interactions, is biphasic, occurs with most stimulators of the enzyme (PGE, Gpp(NH)p) but not F^-, does not operate directly on the catalytic components, and is believed due to a change in membrane conformation. Soluble thyroid membrane cyclase has been prepared to check these effects in a simpler system.

PHS-6040
(12-75)

Project No. Z01 AM 45015-06 CEB

Project Description:

Polycations, including ribonuclease A, ribonuclease S protein and peptide, spermine, spermidine, and polylysines, enhance unstimulated and stimulated adenylate cyclase activity of beef thyroid membranes at low concentrations and inhibit these activities at high concentrations. Peak polylysine stimulation occurs with degrees of polymerization of 6 to 14, and for large polymers a potency limit for this maximum is reached at 4×10^{-5}M expressed as lysine residues. Both enhancement and inhibition appear to be due to charge-charge interactions and are abolished by KCl. Polyanions are inhibitory only. The biphasic effect of polycations is seen on basal cyclase activity, occurs with prostaglandin E_1, and 5'-guanylylimidodiphosphate-stimulated cyclase, but is most striking with thyrotropin. There is little enhancement of F^--activated cyclase. The enhancement is not sensitive to changes in pH, Mg^{2+}, or regenerating system and does not correlate with the stability constants between polycations and ATP. We suggest that the polycation effect is a general, electrostatic effect on membrane conformation and is not restricted to a particular receptor domain. Similar findings have been obtained in intact adrenal and Leydig tumor cells in culture as well as in membranes derived therefrom. In addition, we have succeeded in obtaining "soluble" adenylate cyclase from thyroid membranes by the use of neutral detergents and find that certain of the catalytic properties of this enzyme differ from the membrane enzyme.

Publications:

1. Wolff, J. and Cook, G.H.: Charge effects in the activation of adenylate cyclase. J. Biol. Chem. 250: 6897-6903, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45016-06 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Thyroid Hormone Secretion

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

PI:	J. Wolff	Medical Director	CEB NIAMDD
	B. Bhattacharyya	Visiting Associate	CEB NIAMDD
	F. Cortese	Postdoctoral Fellow	CEB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	2.2	.4

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

Factors affecting the binding of antimitotic agents to tubulin have been obtained in detail. Anions, and in particular tartrate, markedly enhance the rate of colchicine binding (a paradoxically slow process) and not dissociation of ligand. This permits accurate estimates of the binding constant which attains a maximum of $3 \times 10^{7} M^{-1}$. Binding rates for podophyllotoxin and vinblastine are not significantly altered. No conformational changes were detected and it is concluded that effect of the anion is local, near the binding site.

Podophyllotoxin binds rapidly (K_m 1 x 8 x $10^{6} M^{-1}$), is entropy driven, and binds with lower activation energy than colchicine. Podophyllotoxin and colchicine binding are mutually competitive but tropolone interacts only with colchicine binding. Each ligand has two attachment points only one of which is shared by both ligands.

PHS-6040
(12-75)

Project Description:

The factors governing the binding of antimitotic agents to tubulin, and the role of this binding on self assembly to microtubules have been studied further in much greater detail.

The rate of colchicine binding to tubulin, which is normally slow, could be enhanced by various anions. Among the inorganic anions tested, only sulphate was effective. The organic anions include mostly dicarboxylic acids, among which tartrate was the most effective. This rate effect on colchicine binding occurred only at low concentrations of colchicine ($< 0.6 \times 10^{-5}$M). The rate increase for sulfate and L(+) tartrate is $\sim$2.5 fold at 1.0 mM and plateaus at a limiting value of $\sim$ four-fold at 100 mM. The overall dissociation rate of the colchicine from the complex, which includes both the true dissociation rate and irreversible denaturation of tubulin, was not influenced by 1.0 mM tartrate. The affinity constants for colchicine determined from these rate constants are $8.7 \times 10^{6}M^{-1}$ and $2.1 \times 10^{7}M^{-1}$ in the absence and the presence of 1.0 mM L(+) tartrate. The limiting value is $3.2 \times 10^{7}M^{-1}$. The affinity constant calculated from the steady state measurements is $3.2 \times 10^{6}M^{-1}$ with or without anions. The binding of other ligands like podophyllotoxin, vinblastine and 1-anilino-8-naphthalene sulfonate to tubulin was not affected by tartrate. No major conformational changes resulting from anion treatments could be detected by CD, intrinsic fluorescence, partition between the 6S and 36S moieties, or the ability to polymerize. We conclude that anions must have a local effect at or near the binding site.

The binding of [^{3}H]podophyllotoxin to tubulin, measured by a DEAE cellulose filter paper method, occurs with an affinity constant of $1.8 \times 10^{6}M^{-1}$ (37° at pH 6.7). Like colchicine, $\sim$0.8 moles of podophyllotoxin are bound per mole of tubulin dimer, and the reaction is entropy-driven (43 cal deg^{-1} mol^{-1}). At 37° the association rate constant for podophyllotoxin binding is $3.2 \times 10^{6}M^{-1}h^{-1}$, $\sim$8-9 times higher than for colchicine; this is reflected in the activation energies for binding which are 14.7 kcal/mol for podophyllotoxin and 20.3 kcal/mol for colchicine. The dissociation rate constant for the tubulin podophyllotoxin complex is 1.9 h^{-1}, and the affinity constant calculated from the ratio of the rates is identical to that obtained by equilibrium measurements.

Podophyllotoxin and colchicine are mutually competitive inhibitors. This can be ascribed to the fact that both compounds have a trimethoxyphenyl ring and analogues of either compound with bulky substituents in their trimethoxyphenyl moiety are unable to inhibit the binding of either of the two ligands. Tropolone, which inhibits colchicine binding competitively, has no effect on the podophyllotoxin-tubulin reaction. Conversely, podophyllotoxin does not influence tropolone binding. Moreover, the tropolone binding site of tubulin does not show the temperature and pH lability of the colchicine and podophyllotoxin domains, hence this lability can be ascribed to the trimethoxyphenyl-binding region of tubulin. Since podophyllotoxin analogues with a modified

B ring do not bind, it is concluded that both podophyllotoxin and colchicine each have at least two points of attachment to tubulin and that they share one of them, the binding region of the trimethoxyphenyl moiety.

Publications:

1. Wolff, J. and Bhattacharyya, B.: Microtubules and thyroid hormone mobilization. Ann. N. Y. Acad. Sci. 253: 763-770, 1975.

2. Bhattacharyya, B. and Wolff, J.: Membrane-bound tubulin in brain and thyroid tissue. J. Biol. Chem. 250: 7639-7646, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45017-02 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Mechanism of Action of Lithium

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

PI: J. Wolff Medical Director CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.1	.4

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

The mechanism of action of Li^+ in manic depressive psychosis is not understood. Because side reactions to this cation are easier to study we have used adrenal secretory responses to investigate Li^+ effects. Two mM LiCl causes significant inhibition by ACTH-stimulated steroid secretion. With preincubation the required concentration can be reduced to 1 mM.

PHS-6040
(12-75)

Project No. Z01 AM 45017-02 CEB

Project Description:

The mechanism of action of Li^+ in manic depressive psychosis is not understood. Because side reactions to this cation are easier to study we have used adrenal secretory responses to investigate Li^+ effects. Two mM LiCl causes significant inhibition by ACTH-stimulated steroid secretion. With pre-incubation the required concentration can be reduced to 1 mM. Using colchicine, preliminary findings suggest that Li^+ may act by stabilizing microtubules. Direct studies of this are under way.

Publications:

1. Wolff, J.: The endocrine effects of lithium. Neuropsychopharmacology. 359: 621-628, 1974.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45018-01 CEB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Adenylate Cyclase and Other Extracellular Products of B. pertussis: Effects on Mammalian Cells

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

PI:	E. L. Hewlett	Clinical Associate	CEB NIAMDD
	J. Wolff	Medical Director	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAO/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.2	.4

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

Bordetella pertussis organisms exhibit adenylate cyclase in four compartments: 1) soluble, in culture medium, ∿20% of total; 2) associated with intact cells and measured with exogenous ATP (20-45%); 3) extracytoplasmic cyclase not measured by exogenous ATP but sensitive to trypsin (40-60%); 4) intracellular cyclase (10%). Enzyme from compartment 1 has been purified and has a molecular weight of 60,000, is a single subunit and is not sensitive to α-keto acids.

PHS-6040
(12-75)

Project No. Z01 AM 45018-01 CEB

Project Description:

Culture medium of exponentially growing Bordetella pertussis (strain 114) contains significant quantities of soluble (100,000 x g x 1 hour) adenylate cyclase. The enzyme has been purified by chromatography on DEAE-cellulose, and Sephadex G-200. The purest material yielded a single band on SDS disc gel electrophoresis. It is heat labile, has a temperature optimum of 30°C, a pH optimum of pH 7 to 8, a K_m for ATP of 0.4 mM, and requires Mg^{2+} for maximum activity. The molecular weight, by SDS disc gel electrophoresis and sucrose density gradient, is approximately 70,000. The enzyme is markedly inhibited by fluoride and weakly inhibited by monovalent salts, but its activity is not altered by α-keto acids or non-substrate nucleoside triphosphates. Thus, by its presence in the culture supernatant, its smaller molecular weight and its insensitivity to α-keto acids and nucleotides, this enzyme differs from the bacterial adenylate cyclase previously described. In addition, there is an extracytoplasmic adenylate cyclase bound to the intact organisms which forms [^{32}P]cAMP from exogenous [α^{32}P]ATP (200-1200 nmol cAMP formed/min/g wet weight) and which comprises 20-45% of the total adenylate cyclase activity. In contrast, only 1.7 and 2.4% of the total cell malate dehydrogenase [EC1.1.1.37] and alkaline phosphatase (EC3.1.3.1], respectively, are detectable in the intact cell. Trypsin treatment of intact organisms destroys 96% of the extracytoplasmic adenylate cyclase, but does not reduce the total cell malate dehydrogenase or a small pool of intracellular adenylate cyclase. Four compartments of adenylate cyclase in B. pertussis are proposed: A) soluble enzyme in the culture supernatant (up to 20% of the total activity); B) enzyme associated with intact cells and measureable without cell disruption (20-45%); C) extracytoplasmic enzyme sensitive to trypsin, but not measureable in intact cells at standard substrate concentrations (40-60%); and D) intracellular enzyme (less than 10%). Several commerical pertussis vaccines have been found to contain adenylate cyclase activity, probably reflecting the activity of the extracytoplasmic enzyme associated with and released from the whole cells in the vaccine.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 45019-01 CEB

PERIOD COVERED: July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Peroxidase Reactions

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

PI:	J. Pommier	Visiting Associate	CEB NIAMDD
	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD
OTHER:	J. Nunez	Director of Research	C.N.R.S.

COOPERATING UNITS (if any)

Unité de Recherche sur la Glande Thyroïde et la Régulation Hormonale, INSERM, Bicêtre, France

LAB/BRANCH: Clinical Endocrinology Branch

SECTION: Endocrine Biochemistry

INSTITUTE AND LOCATION: NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.6	.2

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

The interaction of sulfhydryl compounds and of diiodotyrosine with various peroxidases was studied. The ability of peroxidases to bind these ligands formed the basis for a method of purification of lactoperoxidase by affinity chromatography.

PHS-6040
(12-75)

Project Description:

Sulfhydryl compounds (R-SH) inhibit the enzymatic activity of peroxidases. They interact with the heme iron of the enzyme as evidenced by a red shift of the Soret band upon addition of R-SH (Cys or glutathione) to a solution of lactoperoxidase (LPO) or horse radish peroxidase (HPO).

In the presence of R-SH (or of H_2O_2 which behaves similarly) LPO and thyroid peroxidase (TPO) also bind diiodotyrosine (DIT). DIT attaches itself not to the heme, but to the protein. (No shift of the Soret band is observed.) It was shown by equilibrium dialysis that DIT (2×10^{-5}M) binds to LPO only after addition of R-SH.

The ability of peroxidases to bind R-SH and DIT was used to develop methods for the isolation and purification of peroxidases by affinity chromatography. Glutathione and DIT were covalently attached to agarose. Both of these polymers bind LPO, TPO, and chloroperoxidase. The binding to DIT-agarose was very strong, even in the absence of R-SH (probably due to the high DIT concentration). In the presence of R-SH, it was so strong that complete elution became difficult. Elution of the peroxidase from glutathione-agarose can be carried out with any substrate (e.g., iodide or guaiacol), each substrate requiring a different pH range. Elution from DIT-agarose can be carried out by raising the ionic strength.

A batch procedure was used for the isolation of LPO from milk by adsorption on DIT-agarose.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 45020-01 CEB

PERIOD COVERED: July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Synthesis of Thyroxine-transport Proteins

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

PI:	M. C. Gershengorn	Clinical Associate	CEB NIAMDD
	D. Glinoer	Postdoctoral Fellow	CEB NIAMDD
	J. Robbins	Chief, Clin. Endo. Branch	CEB NIAMDD
OTHER:	A. Dubois	Guest Worker	DDB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH: Clinical Endocrinology Branch

SECTION: Endocrine Biochemistry

INSTITUTE AND LOCATION: NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.9	1.6

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

Dispersed rhesus monkey hepatocytes were obtained by in situ liver perfusion with collagenase, hyaluronidase and EDTA. Secretion of TBG into the incubation medium was demonstrated by radioimmunoassay, and was linear for 19 hours. De novo synthesis of TBG was shown by incorporation of ^{14}C-leucine. Hepatocytes obtained from monkeys in which estradiol-containing pellets had been implanted showed a 2.6 fold increase in TBG production (3.5 ng/10^7 cells/hr) compared to control. In vivo metabolism of ^{125}I-TBG in estradiol-treated monkeys showed a similar increase in TBG production rate (3.8 mg/day, or 3.2 times control). TBG synthesis was also shown in a monkey hepatocarcinoma cell culture, and its control by thyroxine at physiological concentrations was demonstrated.

PHS-6040

Project Description:

Circulating levels of thyroxine-binding globulin (TBG), the major thyroxine transport protein, are affected by estrogens and other hormones in man and subhuman primates. In order to identify the locus of TBG synthesis and to investigate its possible hormonal control, we undertook a study of TBG biosynthesis in monkey hepatocytes. Dispersed cells were obtained by *in situ* liver perfusion with collagenase, hyaluronidase and EDTA. Conditions for optimum cell survival and incorporation of radioactive leucine into newly synthesized proteins were defined. Incubation medium, cytosol and a particulate fraction (extracted with digitonin) were analyzed for TBG. After extensive dialysis and purification by affinity chromatography, newly synthesized TBG was identified by specific double antibody immunoprecipitation and by immunodiffusion and immunoelectrophoresis with autoradiography. Newly synthesized TBG was present after 4 hours of incubation. After 6 h, the total TBG synthesized had increased to 150% of the 4 h value, while the fraction present in the medium had increased to 300%, indicating probable TBG secretion.

To study the effect of estrogen on TBG synthesis and secretion, we measured TBG by radioimmunoassay and by incorporation of radioactive leucine in the incubation media of hepatocytes isolated from rhesus monkeys treated with β-estradiol (E_2) and from untreated monkeys. Silastic pellets containing E_2 implanted under the skin 2-4 weeks prior to sacrifice resulted in an increase of serum TBG from 19.0 ± 1.0 to 39.8 ± 4.0 µg/ml (mean ± SD) by 14 days. Hepatocytes were isolated, and TBG was measured in the incubation medium by a radioimmunoassay which could detect 0.1 ng monkey TBG in the presence of horse serum. Recovery experiments showed no degradation of secreted TBG. Newly secreted TBG accumulation increased linearly for 19 hr in both groups, and the production rate by hepatocytes from E_2-treated monkeys (3.53 ng/10^7 cells/hr) was 2.6 fold greater than control (1.35 ng/10^7 cells/hr). *De novo* TBG synthesis, shown by incorporation of radioactive leucine into immunoprecipitable TBG in the medium, was increased 3 fold by prior E_2 treatments. These experiments demonstrate increased TBG synthesis and secretion by hepatocytes isolated from monkeys treated with estrogen.

We have also studied ^{125}I-TBG metabolism *in vivo* in normal and estrogen-treated rhesus monkeys. The fractional turnover rates of TBG were similar before and after 4 weeks of E_2 (0.264 *vs* 0.247/day). The distribution volume of TBG, determined by 2 independent methods, increased from 89.8 ± 29.3 ml/kg b.w. to 114 ± 28.4 within 1 day after E_2 implantation. The metabolic clearance rate was also increased (61.5 ± 9.8 *vs* 80.2 ± 16.5 ml/day), possibly as the result of the increased distribution volume. The production rate of TBG, calculated from the metabolic clearance and the serum TBG levels, was increased 3.2 fold from 1.19 ± 0.27 to 3.77 ± 0.92 mg/day. These results indicate that the major effect of estrogen on TBG metabolism is a rapid stimulation of hepatic TBG synthesis.

Additional studies have been carried out in cell cultures in order to provide an *in vitro* system to study TBG control. TBG synthesis and secretion were demonstrated in a continuous cell culture line of Rhesus monkey hepatocarcinoma cells. The cells were shown to survive and grow normally for up to 5 days in the absence of serum, thus permitting study of TBG production in chemically defined media. TBG was identified by its ability to bind thyroxine (T_4) and by immunoelectrophoresis, and quantitated by radioimmunoassay. TBG accumulation in the media was linear for up to 48 hours. T_4, added to the cell culture medium, induced a biphasic response in TBG secretion. There was a progressive increase in TBG accumulation from 10^{-14}M to 10^{-11}M T_4. TBG accumulation decreased from the maximum at T_4 greater than 10^{-10}M, and was depressed below control at T_4 greater than 10^{-8}M. These results indicate that T_4 regulates the synthesis and secretion of TBG in hepatocarcinoma cells, and this is one of few *in vitro* systems to respond to physiological T_4 concentrations ($\sim 10^{-11}$M). Preliminary studies have also demonstrated a 1.5 fold increase in TBG in the medium of cells incubated with β-estradiol (2×10^{-6}M).

Publications:

1. Glinoer, D., Gershengorn, M. C. and Robbins, J.: Thyroxine-binding globulin biosynthesis in isolated monkey hepatocytes. *Biochim. Biophys. Acta* 418: 232-244, 1976.

ANNUAL REPORT: 1975 - 1976

DIABETES BRANCH

Studies in our laboratory over the last several years have indicated that the insulin receptor undergoes major changes in affinity and in concentration in a variety of biologically relevant circumstances *in vivo* and *in vitro*. A major goal of the current research is to characterize these changes, to relate them to changes in target cell sensitivity, experimentally and in disease states in man, and to define some of the molecular mechanisms involved. During the last year, this work has been extended in many laboratories both to other hormone receptors and to other biological systems. Further impact of the work is manifest by the large number of invitations to members of the Branch to lecture, to organize symposia, and to write chapters and reviews in books and journals, as well as the large number of excellent applicants from abroad to work with investigators in the Branch.

1. Negative Cooperativity of the Insulin Receptor

The insulin receptors appear to be a homogeneous set of receptors; when occupied by insulin, the receptors undergo site-site interactions with one another producing a decrease in the affinity for hormone. When the insulin receptors were solubilized from the membrane, they retained the same affinity and the same negatively cooperative site-site interactions indicating that these are intrinsic properties of the insulin receptor itself and do not depend upon their being in the membrane. Extensive analysis of the data by a variety of models and computer simulations has led to new relationships between receptor occupancy and changes in affinity. Among the predictions of the model is that if the shift from the highest to the lowest affinity is quantal, then the insulin receptor in the unoccupied state exists as a tetramer and that occupancy by insulin results in formation of receptor monomers. Recently it was observed that when the soluble receptor is unoccupied, it is recovered as a single peak following gel filtration; when it is partially occupied by insulin, a second peak of activity is noted which is approximately 4-fold lower in molecular weight, and is present in the expected proportions. Thus we have experimental evidence to suggest that a tetramer-monomer shift, which was one of the alternatives presented in the theoretical model, is the actual molecular concomitant of the negative cooperativity. Other studies on the characterization of the insulin receptor show that modification of the microfilaments produces a time-dependent loss of insulin and growth hormone receptors on the cell surface without any effects on the affinity whereas anti-microtubular preparations have no effect. Deuterium oxide, a stabilizer of microtubules and of other proteins produced a significant fall in the affinity of the insulin receptor largely by producing acceleration of the dissociation rate of insulin from its receptors (De Meyts, Ginsberg, Van Obberghen, Kahn).

2. Insulin Receptors in the Insulin Resistance of Obesity

We have previously shown in obese animals that there is a substantial decrease in the concentration of insulin receptors on cells throughout the body and that the magnitude of the decrease in receptor concentration is related to the degree of hyperinsulinemia, that is, to the circulating level of insulin in the basal state and to the degree of insulin resistance. We found a decrease in the concentration of insulin receptors in obese people by studying the receptors on circulating monocytes, and the decrease was proportional to the degree of insulin resistance and hyperinsulinemia. In the occassional obese patients who were not insulin resistant or hyperinsulinemic, insulin binding to receptors was entirely normal. When the hyperinsulinemic obese patients were dieted for several weeks, circulating insulin levels fell and receptor concentrations were restored to normal though the patients were still markedly overweight. In both the fed and dieted states the concentration of insulin receptors was inversely related to the basal levels of circulating insulin. In comparison with obese mice, the receptors in obese people are distinctly more sensitive to elevations in the ambient insulin concentration than are receptors in mice, which is consistent with many observations that people in general are more sensitive to insulin than are rodents. An unusual finding was that when obese hyperinsulinemic patients were fasted for 24-72 hours the insulin levels fell but the receptor concentrations did not rise. However, by 48-72 hours receptor affinity increased about five-fold but only at low levels of receptor occupancy. This would have the effect of restoring insulin sensitivity to low levels of insulin while retaining insulin resistance at higher concentrations of ambient insulin. Most important, these findings indicate that metabolic events *in vivo* result in major shifts in receptor affinity which complement changes in receptor concentration as devices for regulating target cell sensitivity. Present studies are aimed at characterizing the molecular mechanisms for this acute increase in receptor affinity (Bar, Kahn, Gorden).

3. Insulin-Mediated Loss of Insulin Receptors

Previous studies have shown that insulin directly affects the concentration of its own receptors on cells (IM-9 lymphocytes in culture). Recent studies have shown that the magnitude of the fall in insulin receptors produced by six different insulins that varied widely in biological potencies was directly predicted by their biological potency, which is also proportionate to their ability to bind to the insulin receptor. Thus insulin produces the loss of the receptors by first binding to the insulin receptor. The loss of receptors appears to be entirely through an acceleration of the normal degradative process for receptors rather than any effect on receptor synthesis. The loss of receptors appears to require that cellular functions be intact since brief pretreatment of the cells with cycloheximide prevents insulin-mediated loss of the insulin receptor. A reduction in temperature to below 30° also markedly decreases the insulin mediated loss of receptors. Avian erythrocytes, which have normal insulin receptors but lack much of the normal cytoplasmic machinery of the cell do not lose their insulin receptors upon exposure to insulin. Removal of the insulin is followed within hours by restoration of receptors; cycloheximide blocks the return of the receptors (Kosmakos, Kahn, Roth).

4. Insulin Interaction with Macrophages

As a corollary of the study of insulin receptors in human monocytes, we have found insulin receptors on macrophages from the spleens of normal mice as well as on macrophages of a mouse cell line in culture. Further we show that insulin produces a biological effect in these cells at low physiological concentrations of insulin (10^{-10}M): the ability of these cells to kill (antibody-coated red cells) is inhibited by insulin in a dose-dependent fashion. These studies suggest that the insulin receptors of monocytes in humans not only bind insulin but also mediates a biologically relevant cell process (Bar, Koren, Kahn).

5. The Role of Growth, Differentiation, and Cyclic AMP on the Concentration of Insulin Receptors

In collaboration with scientists of the National Cancer Institute, it was found that normal mouse fibroblasts (Balb 3T3) have many fewer insulin receptors per cell or per unit of cell surface when growing than when stationary and there was a strong correlation between the density of cells per culture dish and the concentration of insulin receptors. Transformed cells derived from the Balb 3T3 line have fewer insulin receptors than do the non-transformed cells and again there was a strong positive correlation between the density of the cells and the complement of insulin receptors. Part of the effect of cessation of growth to increase the concentration of insulin receptors could be ascribed to the rise in the levels of cyclic AMP; it is known that the cessation of growth and contact inhibition appears to be associated with the accumulation of intracellular cyclic AMP. Cyclic AMP analogues as well as inhibitors of cyclic AMP degradation produced a 2-fold increase in insulin receptors both in fibroblasts and in IM-9 lymphocytes. Cyclic AMP had no effect on growth hormone receptors of the IM-9 lymphocytes. Thus an additional important influence on the concentration of receptors appears to be the state of growth and differentiation of the cell and that cyclic AMP may be one of the important modifiers of the concentration of insulin receptors (Thomopoulos, Kosmakos, Roth, Pastan, Lovelace).

6. Growth Hormone Modulation of Growth Hormone Receptors

IM-9 lymphocytes, in addition to their insulin receptors, have receptors for human growth hormone. We have previously reported that growth hormone regulates the concentration of its own receptors; growth hormone receptors are even more sensitive to growth hormone than are insulin receptors to insulin, and in every case the effect was specific for the homologous hormone. Growth hormone analogues produce loss of the insulin receptor in proportion to their ability to bind to the growth hormone receptor. Prolonged occupancy of only a minority of sites is capable of producing a substantially greater loss of receptors. Thus 2 ng/ml of growth hormone which occupied about 10% of the receptors at steady state after 90 minutes produces about a 30-40% loss of insulin receptors upon prolonged exposure to hormone. At very high concentrations of growth hormone about 80% of the receptors are lost and further increases in growth hormone concentration produce further increases in receptor occupancy but no further loss of receptors (Lesniak, Roth).

7. Radioreceptor Assay of hGH

The growth hormone receptors on IM-9 lymphocytes have been used to develop a radioreceptor assay for human growth hormone. With this assay we have shown that the larger forms of circulating growth hormone (big GH) have reduced affinity for the growth hormone receptor and further that the circulating growth hormone components in acromegalic patients are moderately more reactive than the comparable components from normal subjects. Research in the field of human growth hormone has been badly hampered by the lack of sensitive, precise, and specific bioassay. The radioreceptor assay which measures a major component of what we call biological activity, has a sensitivity many orders of magnitude greater than the previous bioassays and is thus a tool for general study of the biological properties of growth hormone and related peptides (Gorden, Eastman, Lesniak).

8. Extreme Insulin Resistance in People

We have studied in detail a group of patients who have severe insulin resistance of no previously known cause. These patients are markedly hyperinsulinemic and insulin resistant, and insulin binding to their circulating monocytes is markedly reduced. The degree of reduction of insulin binding to receptors on the patients' monocytes is directly related to the severity of the observed clinical insulin resistance. In one of these groups of patients, it was found that they have circulating antibodies which are directed specifically against the insulin receptor. Serum or highly purified immunoglobulin preparations from these patients will react with normal insulin receptors and cause a markedly impaired binding of insulin. The mechanism for the impairment of binding differs among the different anti-sera; one patient's sera act predominantly to decrease the available number of receptors whereas another patient's anti-sera appear to work entirely by impairing the affinity of the receptor for insulin. Sera from a third patient showed a combination of these properties. In addition to impairing insulin affects, these anti-sera *in vitro* have variable degrees of insulinomimetic activity. Thus the anti-sera were capable of stimulating glucose oxidation in isolated fat cells and this effect was similar to that produced by insulin itself. Anti-sera varied widely in their relative ability to block insulin effects and to mimic insulin effects. Thus these anti-sera are the first specific antagonists (and partial agonists) of insulin. Modifications of the cell surface by enzymatic treatment alters insulin binding somewhat differently than antibody binding suggesting that the antibodies are binding to sites on the receptor that are not identical to the binding site of insulin. In addition to providing an important advance in our understanding of diseases of target cell sensitivity, these anti-sera provide unique probes of receptor structure and function. Additional studies indicate that patients who undergo remission of their insulin resistance also have a disappearance of these antibodies. The antibodies are largely IgG; one patient also had a minor component of IgM. All of the activity of the sera is lost on the addition of anti-IgG antibodies. Since part of the activity is lost with anti-kappa chain and part with anti-lambda chains, the antibodies are of polyclonal origin. The inhibitory activity is retained in the $F(ab)_2$ portion of a pepsin treated immunoglobulin preparation (Kahn, Flier, Jarrett, Gorden, Roth).

We have labeled a purified gammaglobulin preparation from one of these patients with ^{125}I. When the labeled gammaglobulin was exposed to cells that are rich in insulin receptors, the specific antibodies for the insulin receptors bind to the cells. This anti-receptor antibody was eluted from the cells by brief exposure to low pH. The specific activity of the ^{125}I-antibody was thereby enhanced by about 50-fold. When the purified labeled antibody was exposed to insulin receptors, its binding was competed for by gammaglobulin preparations from the patient herself, as well as by sera from other patients with this disease. In addition, the binding of the purified labeled anti-body was competed for by insulin. The ability of an insulin to inhibit the binding of the labeled antibody was directly proportional to the ability of that insulin to bind to the insulin receptor suggesting that the antibody binding site and the insulin binding site are closely related. This new technique allows not only a more precise characterization of the specificity and nature of the antibody but provides a generalized method for the detection and characterization of anti-receptor antibodies. This technique for preparing a purified labeled antibody allows the detection of specific autoantibodies without necessarily having worked out in detail the specific receptor system. Furthermore, it is enoumously more sensitive and quantifiable than immunofluorescence or other techniques that are widely used now for study of autoimmune disorders (Jarrett, Roth, Kahn).

9. Insulin-Like Peptides

This laboratory has had continuing interest in studying insulin-like peptides that are found in plasma. These peptides all have the biological properties of insulin but do not react with anti-insulin antibodies. Using the specific receptors on liver plasma membranes that bind these insulin-like peptides strongly, the characteristics of this receptor have been studied in detail and contrasted with those of the insulin receptor. Furthermore, it has been shown that multiplication stimulating activity (MSA) produced by certain rat liver tumors is indistinghishable from non-suppressible insulin-like activity (NSILA-s) which is present in human plasma. The only other insulin-like peptide that reactsin the system is somatomedin A which has a definite but weak reactivity for the NSILA-s - MSA receptor. Furthermore, it has been shown that these insulin-like peptides are also very potent inhibitors of the insulin-specific protease. In fact they are much more potent than insulin in inhibiting the degradation of ^{125}I-labeled insulin (Rechler, Megyesi, Kahn, Roth).

10. Radioreceptor Assay of NSILA-s

Using the new radioreceptor assay for NSILA-s, we have shown that one-third to one-half of patients with hypoglycemia associated with a non-islet cell tumorshave elevated levels of NSILA-s which account for their hypoglycemia. Furthermore, we have found that between 90 and 95% of the total NSILA-s in plasma is bound to one or more plasma proteins. The concentration of free NSILA-s in plasma of patients with tumor hyperglycemia has an insulin-like potency comparable to the insulin-like potency of patients with insulinomas associated with hypoglycemia. Further studies have shown that intravenous injection of growth hormone produces a prompt multi-fold rise in plasma NSILA-s

associated with a fall in free fatty acids and an occassional fall in the glucose. We suspect that this acute rise in plasma NSILA-s accounts for the well-known early insulin-like effects of growth hormone *in vivo*. Depressed levels of plasma NSILA-s were found in hypopituitary patients and in patients with anorexia nervosa. In patients with hypopituitarism, plasma NSILA-s was restored to normal by hGH, and refeeding of the anorexia nervosa patients likewise restored their plasma NSILA-s concentrations (Kahn, Megyesi).

11. Biological Activity of Insulin-Like Peptides

Extensive work has been done to isolate and purify the insulin-like peptide, MSA or multiplication stimulating activity, that is produced by rat hepatoma cells growing in serum-free medium. The peptides stimulate cell division and thymidine incorporation in chicken as well as human fibroblasts. In addition a novel receptor has been described that binds MSA and insulin nearly equally which is in contrast to the two previously described receptors, namely the insulin receptor (which binds insulin several hundred times better than MSA and NSILA-s) and the NSILA-s receptor (which binds MSA and NSILA-s many times better than it binds insulin). Work is now being extended to demonstrate that this novel receptor is in fact the receptor that mediates the growth promoting effects of these insulin-like peptides and insulin itself (Rechler, Podskalny).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47000-03 DB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cooperative Mechanisms Regulating Receptor's Affinity for Insulin

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

PI:	P. De Meyts	Guest Worker	DB	NIAMDD
	E. Van Obberghen	Guest Worker	DB	NIAMDD
	J. Roth	Medical Director	DB	NIAMDD
	G. Knott	Computer Specialist	LSMM	DCRT

COOPERATING UNITS (if any)

Division of Computer Research and Technology, Laboratory of Statistical and Mathematical Methodology

LAB/BRANCH

Diabetes Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0

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

We have continued to investigate this property of insulin receptors known as negative cooperativity. The affinity of insulin receptors decreases with occupancy, due to site-site interactions which accelerate the dissociation of the insulin-receptor complex. We have done extensive modeling and computer analysis of this system. A simple model has been proposed which predicts the properties of the insulin receptor and provides insights into its minimal structure. The model was also used successfully in computer curve fitting of experimental data. We have also demonstrated that microfilament modifying drugs decrease the concentration of available insulin and growth hormone receptor sites on IM-9 lymphocytes, whereas microtubule modifying drugs are ineffective. In neither case was the negative cooperativity altered.

PHS-6040
(12-75)

Project Description:

We have pursued the study of this property of the insulin receptor known as negative cooperativity, i.e., the affinity of the receptor decreases as occupancy increases, through site-site interactions which accelerate dissociation of insulin from the receptor. We have concentrated on two problems, a) delineating the possible role of the membrane itself, and especially the extent of cytoskeletal control, and b) designing a theoretical model which accounts for the properties of the insulin receptor.

a) Cytoskeletal Control:

Since the cytoplasmic microtubules and microfilaments are involved in the mobility and distribution of surface receptors for immunoglobulins and lectins, we investigated the role of these structures in the binding of insulin and human growth hormone to their receptors on cultured human lymphocytes (IM-9). Cells preincubated with microfilament modifiers, cytochalasins A, B, and D (10 µg/ml), had decreased binding of insulin (32%) and hGH (62%) under steady state conditions, which was not reversed by removing the cytochalasins from the medium and was due entirely to a reduced number of receptor sites on the cell surface. The lost receptors were not detected in the medium, suggesting a redistribution within the cell. The cytochalasins failed to alter the affinity of the hormones for their receptors or the negative cooperativity of the insulin receptor. The antimicrotubule agents (vincristine, vinblastine, colchicine) had no effect. Deuterium oxide, a stabilizer of microtubules and other proteins, decreased the affinity (41%) of insulin for its receptors under steady state conditions and accelerated moderately the spontaneous dissociation of ^{125}I-insulin from its receptors.

Thus, cytochalasin-sensitive microfilaments appear to modulate the exposure of the insulin and growth hormone receptors at the cell surface.

The fact that the negative cooperativity of insulin receptors is unaltered under conditions which disrupt microtubule and microfilaments makes it more likely that the cooperativity is an intrinsic property of the insulin receptor and that site-site interactions are not modulated by surface movements of receptors. This was independently confirmed by B. Ginsberg in our group, which demonstrated that the cooperative properties are intact in the detergent-solubilized receptor.

b) Theoretical Modeling:

Insulin and several other polypeptide hormones induce site-site interactions of the negative cooperative type among their receptors. The average affinity, $\bar{K}$, goes from a high affinity, $\bar{K}_e$, when the receptors are empty, to a low affinity, $\bar{K}_f$, when the receptors are filled (i.e., when the fractional occupancy, $\bar{Y}$, goes from 0 to 1). In previous studies, we have used a plot of $\bar{K}$ against $\bar{Y}$ to display the changing receptor affinity. We now describe a precise mathematical

function which links $\bar{K}$ to $\bar{Y}$ and, with minimal assumptions, predict all the binding properties of the insulin receptor including its subunit structure. According to this model, the fall in affinity due to cooperativity is a simple, linear function of occupancy:

$$\bar{K}_e/\bar{K} = 1 + \frac{1-\alpha}{\alpha}\bar{Y} \quad (1) \quad \text{or} \quad \bar{K} = \bar{K}_e/(1 + \frac{1-\alpha}{\alpha}\bar{Y}) \quad (2)$$

where α is a constant interaction factor equal to $\bar{K}_f/\bar{K}_e$. By substituting $\bar{K}$ for the usual affinity constant into the classical binding equations, one can easily generate the cooperative binding curve and its various transformations (Hill, Scatchard). This model is valid irrespective of whether the individual receptor sites go from $\bar{K}_e$ to $\bar{K}_f$ in a stepwise or quantal fashion. If the shift is, in fact, quantal, the fraction of sites in $\bar{K}_e$ and $\bar{K}_f$ at any occupancy is easily calculated from the average affinity $\bar{K}$. The minimal number of interacting subunits is then obtained by dividing the fraction of sites in $\bar{K}_f$ by the fraction of sites occupied, $\bar{Y}$.

Using the MLAB program (Gary D. Knott, DCRT), we have now used this model extensively for computer simulation and, with excellent results, for curve-fitting of experimental data.

The above model fits actual binding data with great accuracy. The receptor sites undergo a ten-fold drop in affinity upon occupancy with insulin, and the calculated number of interacting subunits of a receptor is four. The number of subunits interacting decreases sharply with increasing occupancy possibly suggesting receptor depolymerization. Furthermore, if the cell responds biologically in proportion to the fraction of sites switched to the low affinity state, $\bar{K}_f$, and not to $\bar{Y}$ as usually assumed, the negative cooperativity in binding results in an amplified biological response at low occupancy and apparent "spare receptors".

Publications:

1. Limbird, L. E., De Meyts, P., and Lefkowitz, R. J.: β-adrenergic receptors: Evidence for negative cooperativity. Biochem. Biophys. Res. Commun. 64: 1160-1168, 1975.

2. De Meyts, P., and Roth, J.: Cooperativity in ligand binding: A new method of graphic analysis. Biochem. Biophys. Res. Commun. 66:1118-1126, 1975.

3. De Meyts, P.: Cooperative properties of hormone receptors in cell membranes. J. Supramolec. Struct. 4: 241-258, 1976.

4. De Meyts, P., Bianco, A. R., and Roth, J.: Site-site interactions among insulin receptors: Characterization of the negative cooperativity. J. Biol. Chem. 251: 1877-1888, 1976.

5. De Meyts, P.: The negative cooperativity of insulin receptors: A model for the regulation of hormone recognition by target cells. In Bassett, E.G., (Ed.): Cell membrane receptors for viruses, antigens, and antibodies, polypeptide hormones and small molecules. Proceedings of the 9th Miles International Symposium, Baltimore, Maryland, The Raven Press, 1976, in press.

6. De Meyts, P.: Cooperative regulation of hormone binding affinity for cell surface receptors. In Bradshaw, R. A. (Ed.) Surface membrane receptors: Interface between cells and environment. Proceedings of a NATO Advanced Study Institute, Bellagio, Italy, Plenum Press, 1976, in press.

7. Simon, J., Freychet, P., Rosselin, G., and De Meyts, P.: Enhanced binding affinity of chicken insulin for receptors in rat liver membranes and human lymphocytes: Relationships to the kinetic properties of the hormone-receptor interaction. Endocrinology, 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47001-05 DB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Receptors on Cultured Human Lymphocytes by Insulin

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

PI:	F. Kosmakos	Guest Worker	DB	NIAMDD
	P. Thomopoulos	Guest Worker	DB	NIAMDD
	J. Roth	Medical Director	DB	NIAMDD
	D. M. Neville, Jr.	Medical Director	LNC	NIMH

COOPERATING UNITS (if any)

Laboratory of Neurochemistry, National Institute of Mental Health

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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

We have previously described that the concentration of insulin receptors on IM-9 lymphocytes is inversely related to the ambient insulin level. This homeostatic regulation of insulin receptor concentration by insulin is dependent upon the duration and concentration of insulin. Insulin analogue studies have shown that the loss of receptors is mediated via the receptor in direct proportion to the bioaffinity of the analogues. The response is temperature dependent and requires protein synthesis. Studies involving protein synthesis inhibitors are now being carried out in order to elucidate the fate of the receptors under our experimental conditions.

PHS-6040
(12-75)

Project Description:

We have extended our studies on the homeostatic regulation of insulin receptor concentrations by insulin in the external milieu. Our previous studies have shown that the steady state concentration of insulin receptors on cells is inversely related to the chronic level of insulin to which the cells are exposed; with humans or rodents *in vivo* or cultured human lymphocytes *in vitro*, up to 75% of the receptors are lost in the presence of high insulin concentrations.

The loss in insulin binding is due to a loss in receptor number and not to altered receptor affinity for insulin or contamination by the ambient insulin. The characteristics of ^{125}I-insulin binding to cells which have been exposed chronically to insulin were similar to those observed with untreated cells. Cells with altered receptor number exhibited the same pH and temperature dependence of binding as well as the phenomenon of negative cooperativity.

The effect of insulin on the insulin receptor is mediated via the receptor itself, since insulin analogues which cannot induce site-site interactions (negative cooperativity) among insulin receptors, induced receptor loss in proportion to the biopotency (and binding affinity) of each analogue.

In experiments with combinations of cycloheximide and insulin, it was found that (1) the normal rate of insulin receptor turnover was slow (1.5% per hour), (2) insulin acted to accelerate (3 to 6-fold) loss of receptors with little or no effect on receptor biosynthesis, (3) the reduced complement of receptors could not be restored to normal except by both removing the insulin and allowing new protein systhesis to occur, and (4) the insulin-mediated receptor loss involved one or more rapidly turning over cellular components, since brief pretreatment of cells with cycloheximide blocked the effect of insulin on receptor loss. In the same cells, cycloheximide pre-treatment did not prevent growth-hormone induced loss of growth hormone receptors. These observations, coupled with the fact that the rate of receptor decrease is proportional to both the ambient insulin level and the biopotency of the analogue suggest that the insulin-mediated receptor response is a cell-mediated process which occurs by accelerated degradation of the insulin receptor and with time, the membrane insulin receptor concentration approaches equilibrium at a new steady state level.

Further evidence that insulin-induced receptor loss requires complex cellular organization was that inhibition of ATP production or reduction in temperature inhibition the effect of insulin on receptor loss. Disruption of microtubules or microfilaments with inhibitors was without effect on the insulin mediated effect. Support for these conclusions comes from studies with avian erythrocytes. These cells have normal insulin receptors but intracellular organelles are sparse, nuclei are pyknotic and protein synthesis is very low; even prolonged exposure of these cells to very high insulin concentrations produced no loss of insulin receptors.

Project No. Z01 AM 47,001-05 DB

In collateral research, the effect of cyclic nucleotides on insulin binding was examined with human cultured lymphocytes. Preincubation of cells with dibutyryl cAMP, 8-bromo-cAMP, or 1-methyl 3-isobutylxanthine (but not 8-bromo-cGMP) elevated insulin binding 2-fold due to an increase in receptor concentration without any effect on receptor affinity for insulin. The increase in receptors was dose-dependent, required 8 hours of preincubation, reached a plateau by 24 hours, and was reversed by removal of the nucleotide. The effect of cAMP was not observed with human growth hormone binding and was independent of changes in cell cycle.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47002-02 DB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Insulin Receptors in Diverse Tissues

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

PI:	B. Ginsberg	Surgeon	DB NIAMDD
	M. Muggeo	Guest Worker	DB NIAMDD
	C. R. Kahn	Senior Surgeon	DB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Diabetes Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

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

Detergents have been widely used to solubilize tissue receptors. We have used detergent to solubilize insulin receptors from avian erythrocytes. The detergent solubilized insulin receptor retains all of the binding properties shown with cell and membrane preparations. In particular negative cooperativity was demonstrated and may be associated with a size change of the receptor. We are presently attempting to further characterize this size change. We are extending the study of insulin receptors to include other species along the phylogenetic line in order to determine the evolution of the receptor.

PHS-6040
(12-75)

Project No. Z01 AM 47,002-02 DB

Project Description:

We have previously shown that the insulin receptor of the turkey erythrocyte is very similar to that of mammals. We have extended these studies to the soluble turkey receptor and have demonstrated that the receptor, freed from the membrane, retains all binding properties including negatively cooperative site-site interactions and that these interactions are associated with an apparent change in receptor size. Receptors solubilized from purified erythrocyte membranes with 1% Triton X-100 were not retained by 0.2 μ filters, did not sediment at 400,000 x g, and eluted from Sepharose 6B with a molecular weight of 300,000. Specificity for insulin analogues, temperature dependence of binding and the equilibrium constants were identical to those found with intact erythrocytes. Scatchard analysis yielded a curvilinear plot and kinetic experiments revealed that the initial dissociation rate in the presence of insulin (1 μg/ml) was 2-fold increased over that caused by dilution alone, indicating that there were site-site interactions. Incubation of receptor with 25 ng/ml of insulin produced a fall in average affinity consistent with a shift of 35% of receptors to the low affinity state. Assay of the effluent of a Sepharose column in the presence of this concentration of insulin revealed a shift of about 25% of the receptors to a lower apparent molecular weight (∿ 75,000). These data indicate that the insulin receptor retains its cooperative properties when removed from the membrane and suggest that the site-site interactions are associated with a tetramer ↔ monomer transition.

We have also extended our studies on the evolution of the insulin receptor. We had previously shown that the insulin binding properties of the insulin receptor of a variety of mammalian and avian tissues were virtually identical. We now report that while the binding properties of the insulin receptor of the frog and trout are very similar to those of mammals there are distinct differences. All species studied showed saturable, reversible insulin binding; a variety of natural and chemically modified insulins were bound in direct proportion to their insulin-like biological activity; and all receptors in all species demonstrated negatively cooperative site-site interactions. While the pH for optimum insulin binding for mammalian and turkey receptors was 7.8 - 8.0 the frog erythrocyte bound insulin maximally at pH 7.3 and the trout erythrocyte at 7.6, and while mammalian, turkey and frog insulin binding was always greater at 15° than 22°, trout had equivalent binding at these temperatures. Thus, while insulin binding to vertebrate cells appears to have some invariant properties, such as negative cooperativity and specificity for polypeptides with insulin-like bioactivity, poikilothermic animals seem to have somewhat different insulin binding characteristics. No specific binding of insulin to Escherichia coli (K12) or to spheroplasts formed from these bacteria could be demonstrated.

Publications:

1. Ginsberg, B. H., Kahn, C. R., and Roth, J.: The Insulin Receptor of the Turkey Erythrocyte: Purification and Characterization of the Membrane Bound Receptor. Biochim. Biophys. Acta., in press.

2. Ginsberg, B. H.: The Insulin Receptor: Properties and Regulation. In Litwak, G. (Ed.): Biochemical Actions of the Hormones. New York, Academic Press, Volume 4, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47003-01 DB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Insulin Receptors in Fibroblasts and Their Relationship to Growth and Transformation

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

PI:	P. Thomopoulos	Guest Worker	DB	NIAMDD
	J. Roth	Medical Director	DB	NIAMDD
	I. Pastan	Medical Director	LMB	NCI
	E. Lovelace	Biologist	LMB	NCI

COOPERATING UNITS (if any)

Laboratory of Molecular Biology, National Cancer Institute

LAB/BRANCH

Diabetes Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.75	1.75	1

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

There are insulin receptors in cultured mouse fibroblasts. The characteristics of the mouse fibroblasts are similar to insulin receptors in other tissues. A difference in receptor concentration is found along the growth cycle between normal and transformed cells.

PHS-6040
(12-75)

Project No. Z01 AM 47,003-01 DB

Project Description:

Differences in several aspects of cell membrane structure and function have been described between normal and transformed cells as well as between the growth and stationary phase of normal cells. This study with the normal and transformed mouse fibroblasts was undertaken in order to permit new insight into membrane properties and a correlation of hormone binding with already described transformation-dependent cell membrane functions.

Insulin receptors are well-characterized cell membrane components in several tissues, i.e., adipocytes, hepatocytes, cultured lymphocytes, etc. The characterization studies have been extended to normal (Balb 3T3) and transformed mouse fibroblasts of this cell line. Insulin binds to normal Balb 3T3 cells and the transformed cells. The characteristics of the mouse fibroblasts are similar to insulin receptors in other systems including kinetics of association, negative cooperativity, affinity, pH dependence, and specificity for insulin and insulin derivatives. However, insulin binding is low in growing normal cells and in transformed cells and increased in the stationary normal cells.

Publications:

1. Thomopoulos, P., Roth, J., Lovelace, E., and Pastan, I.: Insulin Receptors in Normal and Transformed Fibroblasts: Relationship to Growth and Transformation. Cell, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47004-01 DB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Insulin Receptors and Insulin-Action in Macrophages

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

PI:	R. S. Bar	Staff Fellow	DB NIAMDD
	H. Koren	Immunologist	LI NCI
	C. Siebert	Bio Lab Tech	DB NIAMDD

COOPERATING UNITS (if any)

Laboratory of Immunology, National Cancer Institute

LAB/BRANCH

Diabetes Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1.5	0.5

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

Insulin receptors were identified on two macrophage populations, the P388D1 cell, and in mixed mononuclear cell preparation from mouse spleen. These receptors were shown to be similar to other well-characterized mammalian receptors for insulin. In these two cell populations insulin has been found to alter the immunologic functions of the macrophages. These cell lines can be used to measure receptor function by following a biological response.

PHS-6040
(12-75)

Project No. Z01 AM 47,004-01 DB

Project Description:

Insulin receptors were identified on two macrophage populations, the $P388D_1$ cell line and in a mixed mononuclear cell preparation from mouse spleen. These receptors are identical to the insulin receptors of circulating human macrophages (monocytes) and other mammalian cells on the basis of pH and temperature dependence, specificity for insulin analogues and kinetics of association and dissociation. In both of these macrophage populations insulin was found to alter immunologic functions, specifically the macrophage-induced antibody mediated cytotoxicity. Insulin at physiological concentrations (10^{-10}M) significantly inhibited the cytotoxicity with increasing inhibition (up to 70%) with increasing concentrations of insulin.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47005-04 DB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Insulin Receptors in Normal and Obese Patients

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

PI:	R. S. Bar	Staff Fellow	DB NIAMDD
	C. Siebert	Bio Lab Tech	DB NIAMDD
	J. Roth	Medical Director	DB NIAMDD
	P. Gorden	Senior Surgeon	DB NIAMDD
	C. R. Kahn	Senior Surgeon	DB NIAMDD
	P. De Meyts	Guest Worker	DB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Diabetes Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2.5	0.5

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

Circulating monocytes are obtained from normal and obese patients. The monocytes have been shown to have receptors for insulin. Using the radio-receptor assay techniques to demonstrate binding of insulin to the patients receptors it has been found that obese patients have a decrease in receptor concentration. Chronic dieting in the obese patient causes an increase in receptor concentration. In the basal, fed state and chronic diet total receptor concentration was inversely related to the patient's circulating plasma insulin level. A 72 hour fast resulted in an increase of receptor concentration only at low concentration of insulin; this binding increase is associated with a change in receptor affinity. These studies are to be extended to other disease states in humans.

PHS-6040
(12-75)

Project Description:

Insulin binding to the insulin receptor has been evaluated in obese patients studied under various dietary conditions. The circulating human monocyte was used as a source of insulin receptors. We found (1) obese patients with significant hyperinsulinemia have a decrease in insulin binding due to a decreased concentration of insulin receptors. (2) In these patients chronic dieting resulted in a fall in plasma insulin and restoration of insulin binding due to an increased receptor concentration. (3) A 72 hour fast resulted in increased insulin binding only at low concentrations of insulin; this binding increase was due to a change in receptor affinity. (4) In the basal, fed state and after chronic diet total receptor concentration was inversely related to the patient's circulating plasma insulin level.

Publications:

1. Roth, J., Kahn, C. R., Lesniak, M.A., Gorden, P., De Meyts, P., Megyesi, K., Neville, D. M., Jr., Gavin, J. R., III, Soll, A. H., Freychet, P., Goldfine, I. D., Bar, R. S., and Archer, J. A.: Receptors for insulin, NSILA-s, and growth hormone: Applications to disease states in man. Recent Progress in Hormone Research. Vol. 31. Proceedings of the 1974 Laurentian Hormone Conference, 1975, pp. 95-139.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47006-01 DB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

In vivo Study of Receptors for Polypeptide Hormones

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

PI:	A. J. Zeleznik	Guest Worker	DB NIAMDD
	J. Roth	Medical Director	DB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0

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

By studying the apparent volumes of distribution of high affinity and low affinity in rabbits, we were able to define a body space which corresponds to insulin receptors. This space has limited capacity since increasing concentrations of unlabeled insulin progressively decreased the distribution volume of the high affinity insulin. In addition, insulin binding *in vivo* is rapid and readily reversible. This reversibility shows that the receptor bound hormone is in rapid equilibrium with the plasma compartment and further suggests that the insulin receptor may function as a reservoir for the circulating hormone, analogous to the plasma binding proteins for steroid and thyroid hormones.

The direct *in vivo* studies reflect the actual number of receptors exposed to hormone under physiological conditions.

This is not a destructive technique. This is really applicable to study of other hormones where target cell is unavailable or unknown.

PHS-6040
(12-75)

Project Description:

Using a novel procedure, we were able to estimate whole body insulin receptor concentration and study some characteristics of the insulin-insulin receptor interaction *in vivo*. When iodinated insulins with low affinity for receptor (guinea pig and proinsulin) were injected intravenously into anesthetized rabbits, the hormone distributed into a volume equivalent to the extracellular fluid space. In contrast, iodinated insulins with high affinity for receptor (porcine, chicken) distributed into a space approximately 2.5 times that of the low affinity insulin. When the occupancy of insulin receptors was increased by prior injection with unlabeled insulin, the distribution volume of the high affinity insulin was progressively reduced; with insulin at a concentration that saturates the receptor (10^{-6}M), the distribution volume of the high affinity insulin equalled that of the low affinity insulin. When unlabeled insulin was injected 5-30 minutes after the injection of the labeled insulins, there was a prompt and substantial rise in the plasma level of ^{125}I-high affinity insulin without a change in the low affinity insulin.

These results demonstrate the existence of a body compartment that corresponds to the insulin receptor. One-half to two-thirds of the insulin outside of the β-cell is receptor bound. The rapid removal of high affinity insulin from the circulation is due to receptor uptake. The magnitude of this uptake is dependent on the number of unoccupied insulin receptors, and further, once insulin is bound to receptors *in vivo*, the receptor bound hormone is in rapid equilibrium with the plasma compartment which suggests that the insulin receptor may function as a reservoir for the circulating hormone, analogous to the plasma binding proteins for steroid and thyroid hormones.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47007-01 DB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Detection and Characterization of Anti-Insulin Receptor Antibodies in Man

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

PI:	J. S. Flier	Surgeon	DB NIAMDD
	C. R. Kahn	Senior Surgeon	DB NIAMDD
	J. Roth	Medical Director	DB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Diabetes Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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

In the present study we have defined a new syndrome composed of insulin resistance and acanthosis nigricans. We have demonstrated that in some of these patients the decrease in insulin binding to its receptors is due to a circulating autoantibody to the insulin receptor. The antibody specifically blocks insulin receptors in a variety of tissues and acts through the $F(ab)_2$ binding sites. Different patients' antisera produce the decrease in binding via different mechanisms. Thus, these antibodies serve as unique probes of both insulin structure and function.

PHS-6040
(12-75)

Project No. Z01 AM 47,007-01 DB

Project Description:

We have defined and characterized the mechanism for the insulin resistance which is seen in several patients with an unusual syndrome of extreme insulin resistant diabetes. This syndrome, which we have formally described, is similar to that reported in a single patient by Dr. J. Field at the NIH in 1961, characterized by variable degrees of glucose intolerance, marked endogenous hyperinsulinemia (10 to 100-fold increased), extreme resistance to exogenous insulin, and variable degrees of the skin condition acanthosis nigricans. The extreme insulin resistance in these patients occurs in the absence of any of the previously known causes of insulin resistance. Studies of the insulin receptor in these patients were carried out using the circulating monocyte, a cell whose insulin receptors are functionally identical to those on more classical target tissues for insulin, and which we believe reflect the state of insulin receptors on other tissues in the body.

Studies of insulin receptors on circulating monocytes in these patients suggest that the insulin resistance is due to a marked decrease in insulin binding to its specific membrane receptors. These patients have insulin binding that ranged from 5 to 60% of control, and the magnitude of the defect in insulin binding correlated well with the severity of the insulin resistante observed clinically.

In three patients with this syndrome who had features of an autoimmune disease, we have found a serum factor which specifically inhibits insulin binding to its receptors from species as diverse as man, mouse, and bird, and tissues as diverse as hepatocyte, adipocyte, fibroblast, placenta, erythrocyte and monocyte.

This circulating inhibitor is an immunoglobulin by multiple criteria. Thus, it is precipitated by 33% ammonium sulfate, migrates with the immunoglobulins on Sephadex G-200 columns and DEAE cellulose chromatography and is entirely removed by immunoprecipitation with specific anti-human immunoglobulins. Further, we have shown that the inhibitory activity resides in the $F(ab)_2$ fraction of the immunoglobulin molecule. Activity is primarily associated with immunoglobulin of the IgG class, but one patient has significant activity in the IgM class as well. Additionally, these antibodies are polyclonal, with both kappa and lambda light chain determinants.

It appears that "anti-receptor antibodies" from different patients affect insulin receptor binding in different ways. Treatment of normal cells with one of the antibodies appears to cause a reduced number of receptors each with normal affinity for insulin. Antibody from another patient appears to reduce receptor affinity, with little or no effect on receptor number. In this case, the main effect appears to be a reduction of association rate for insulin. These different effects suggest that the antibodies are binding to slightly different sites on or near the receptor, making them unique probes of receptor structure and function.

We have found anti-insulin receptor antibodies in 7 of 16 patients with severe insulin resistance in the absence of any previously known causes. While we have not yet screened large numbers of patients to determine the true incidence of these antibodies in disease, we have found no positives among 70 other selected patients studied. Included in this group were patients with juvenile, maturity onset and lipoatrophic diabetes and a wide variety of collagen diseases.

Our intent is to better determine the true incidence of these antibodies in disease, and to use these antibodies as probes of insulin receptor structure and function.

Publications:

1. Flier, J. S., Kahn, C. R., Roth, J., and Bar, R. S.: Antibodies that impair insulin receptor binding in an unusual diabetic syndrome with severe insulin resistance. Science 190: 63-65, 1975.

2. Kahn, C. R., Flier, J. S., Bar, R. S., Archer, J. A., Gorden, P., Martin, M., and Roth, J.: The syndromes of insulin resistance and acanthosis nigricans: Insulin receptor disorders in man. New Engl. J. Med. 294: 739-745, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47008-06 DB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Detection and Characterization of Cell Surface Receptors Using Specific ^{125}I-Receptor Antibodies

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

PI:	D. B. Jarrett	Guest Worker	DB NIAMDD
	J. Roth	Medical Director	DB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Diabetes Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0

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

A new direct method has been developed for the detection and characterization of cell surface receptors for insulin. Specific insulin anti-receptor autoantibodies have been radioactively labeled and purified by cytoadsorption and elution from cells rich in insulin receptors. This new method is generally applicable to the study of other autoimmune diseases.

PHS-6040
(12-75)

Project Description:

Autoantibodies to the insulin receptor, found in some patients with an unusual form of insulin resistant diabetes, specifically inhibit the binding of insulin to its receptor. This inhibitory activity is in the Fab region of these polyclonal IgG molecules. In the present study these antibodies were used to develop a new assay for the insulin receptor. The IgG fraction of one patient's plasma was purified by DEAE chromatography and labeled (1:1) with ^{125}I. The ^{125}I-receptor antibody, which initially represented ∿ 1% of the total ^{125}I-IgG, was purified ∿ 50-fold by selective cytoadsorption and elution. The ^{125}I-receptor antibody was bound to cells with insulin receptors (IM-9 cultured human lymphocytes, rat liver membranes, and avian erythrocytes) and 85% of the bound radioactivity was competitively displaced by porcine insulin. Insulins which differed 300-fold in biological potency inhibited ^{125}I-receptor antibody binding in direct proportion to their ability to bind to the insulin receptor. The only substances other than insulin that inhibited ^{125}I-receptor antibody binding were the purified IgG and $F(ab)_2$ fractions from this patient's plasma, and plasma from other patients with insulin resistance due to anti-receptor autoantibodies. Normal plasma or IgG, plasma with anti-insulin antibodies, and various hormones, including those which have cell surface receptors on these cells, were without effect upon the antibody binding. Cells with a reduced insulin receptor concentration due to a proteolytic treatment with trypsin, or to specific auto-regulation following prolonged incubation with the hormone, also had decreased binding of ^{125}I-receptor antibody. Reduction of hGH receptors was without effect on antibody binding.

Until now the only methods available for measuring cell surface receptors and their antibodies have been by studying the binding and displacement of ^{125}I-labeled purified hormones. By ^{125}I-labeling of the IgG from appropriate sera and selective cytoadsorption, we have developed a precise sensitive new method to detect directly the presence of the receptor and its antibodies.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47009-01 DB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effect of Anti-Insulin Antibodies on Isolated Adipocytes

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

PI:	C. R. Kahn	Senior Surgeon	DB NIAMDD
	K. Baird	Bio Lab Tech	DB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Diabetes Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1

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

We have studied the effect of three different anti-receptor anti-sera on insulin binding and glucose oxidation by isolated adipocytes. All sera inhibited insulin binding by altering receptor affinity. Titers ranged from 1:5 to 1:5000. Some sera also blocked insulin stimulated glucose oxidation, while others stimulated this function. Both activities were retained purified IgG and F(ab)$_2$ fractions of the antibodies. Thus, these antibodies serve as unique probes of both insulin structure and function.

PHS-6040
(12-75)

Project Description:

We have previously reported a syndrome of severe insulin resistance associated with circulating antibodies directed at the insulin receptor. In this project we have used these anti-sera as probes of the insulin receptor of isolated rat adipocytes. All sera blocked ^{125}I-insulin binding to isolated adipocytes and stimulated glucose oxidation. In each case the inhibition of binding observed at 37° was due to a decrease in receptor affinity with no change in receptor number. Fifty percent inhibition of binding occurred with serum dilutions of 1:5 to 1:5000. With one serum maximal stimulation of glucose oxidation occurred with only 10% inhibition of insulin binding; with the second, inhibition of binding and stimulation of glucose oxidation showed superimposable curves; while, the third inhibited insulin binding more effectively than it stimulated glucose oxidation. Cells preincubated with the latter serum in a concentration sufficient to inhibit insulin binding by 80% showed a 50-fold shift to the right in the dose response of insulin stimulated glucose oxidation. Both activities were retained in purified immunoglobulin fractions and in the $F(ab)_2$ fragment of IgG, and were precipitated by antibodies to human IgG but not by antibodies to insulin. When fat cells were exposed to neuraminidase, both insulin and antibody stimulated glucose oxidation decreased in parallel; by contrast, trypsin digestion had a more profound effect on insulin stimulated glucose oxidation. These data suggest that these antibodies bind to different determinants on the insulin receptor making them unique probes of the structure and function of the insulin receptor.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 47010-04 DB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies of Non-Suppressible Insulin-Like Activity (NSILA-s) in Plasma

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

PI:	K. Megyesi	Guest Worker	DB	NIAMDD
	C. R. Kahn	Senior Surgeon	DB	NIAMDD
	J. Roth	Medical Director	DB	NIAMDD
	P. Gorden	Senior Surgeon	DB	NIAMDD
	D. M. Neville, Jr.	Medical Director	LNC	NIMH
	S. P. Nissley	Senior Surgeon	MET	NCI

COOPERATING UNITS (if any)

Laboratory of Neurochemistry, National Institute of Mental Health
Metabolism Branch, National Cancer Institute

LAB/BRANCH

Diabetes Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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

NSILA-s (nonsuppressible insulin-like activity soluble in acid ethanol) is a serum peptide that has insulin-like and growth promoting effects. We have demonstrated that this peptide binds with high affinity to its own receptors and low affinity to insulin receptors. In addition, this peptide has a relatively high affinity for liver plasma membrane insulinases and inhibits insulin degradation. About 90-95% of plasma NSILA-s is bound to a high molecular weight binding protein. Using a radioreceptor assay for NSILA-s we have found elevated levels in some patients with tumor hypoglycemia.

PHS-6040
(12-75)

Project Description:

NSILA-s (non-suppressible insulin-like activity, soluble in acid ethanol) is a serum peptide that has insulin-like and growth-promoting activities. We have demonstrated previously that liver plasma membranes possess separate receptors for NSILA-s and insulin which overlap in their specificities for both peptides and developed a specific and sensitive radioreceptor assay for NSILA-s in plasma. We characterized the properties and specificity of the NSILA-s receptor and compared them to the insulin receptor. Furthermore, of the many growth-promoting peptides that were tested in the NSILA-s receptor assay we found that only multiplication-stimulating activity was fully as active as NSILA-s, and somatomedin A was 10% as active as NSILA-s.

In addition to its insulin-like biological activity and ability to react with insulin and NSILA-s receptors in liver membranes, NSILA-s is also a potent inhibitor of the insulin-degrading system of the liver plasma membranes. The most purified NSILA-s preparation tested (70 mU/mg) was 20-fold more potent that insulin itself in inhibiting ^{125}I-insulin degradation by this enzyme. Other NSILA-s preparations inhibit insulin degradation in rank order of their biopotency and in each case the inhibition appears to be competitive in nature. Furthermore, significant inhibition of insulin degradation occurred at concentrations of NSILA-s similar to those found in plasma.

Studies in plasma showed elevated NSILA-s levels in 5 of 15 patients with extra-pancreatic tumors and hypoglycemia. NSILS-s levels were also elevated in pregnancy and low in hypopituitarism and anorexia nervosa. Within one hour after hGH is given (i.v.) the NSILA-s level rises in hypopituitary patients. In normal subjects, NSILA-s rises after oral glucose and falls with insulin induced hypoglycemia. In plasma at physiologic pH about 90-95% of endogenous NSILA-s is reversibly bound to larger molecules.

Publications:

1. Megyesi, K., Kahn, C. R., Roth, J., and Gorden, P.: Circulating NSILA-s in man: Preliminary studies of stimuli *in vivo* and of binding to plasma components. J. Clin. Endocrinol. Metab. 41: 475-484, 1975.

2. Megyesi, K., Kahn, C. R., Roth, J., Neville, D. M., Jr., Nissley, S. P., Humbel, R. E., and Froesch, E. R.: The NSILA-s receptor in liver plasma membranes, characterization and comparison with the insulin receptor. J. Biol. Chem. 250: 8990-8996, 1975.

3. Kahn, C. R., Megyesi, K., and Roth, J.: Non-suppressible insulin-like activity of human serum: A potent inhibitor of insulin degradation. J. Clin. Invest. 57: 526-529, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47011-03 DB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Interaction of Multiplication Stimulating Activity (MSA) with Target Cells

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

PI:	M. M. Rechler	Senior Surgeon	DB	NIAMDD
	J. M. Podskalny	Biologist	DB	NIAMDD
	S. P. Nissley	Senior Surgeon	MET	NCI
	A. C. Moses	Surgeon	MET	NCI

COOPERATING UNITS (if any)
Metabolism Branch
National Cancer Institute

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1

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

We have studied the interaction of a somatomedin-like growth polypeptide purified from rat liver cell culture medium, multiplication stimulating activity (MSA), with cultured chick fibroblasts, cultured human fibroblasts and epithelial cells. A growth peptide receptor that specifically binds MSA, NSILA-s, and somatomedin A has been identified. Our working hypothesis is that these polypeptides induce DNA synthesis by binding to the growth peptide receptor.

PHS-6040
(12-75)

Project Description:

Multiplication stimulating activity (MSA) is a 10,000 molecular weight polypeptide that potently stimulates cell growth and DNA synthesis, and has weak insulin-like activity. Using MSA purified to homogeneity and labeled with ^{125}I, we have demonstrated MSA binding to a specific receptor in cultured chicken fibroblasts, cultured human fibroblasts, cultured rat liver cells, and rat liver plasma membranes. The binding of ^{125}I-MSA was potently inhibited by MSA itself, and by two closely related polypeptides purified from human serum, somatomedin A and non-suppressible insulin-like activity (NSILA-s). Two types of growth peptide receptors have been distinguished: one in fibroblasts in which insulin effectively competes for MSA binding, and one in hepatocytes in which insulin is virtually inactive. The specificity of the fibroblast growth peptide receptor differs from that of fibroblast insulin receptors. The polypeptides that interact with the growth peptide receptor -- MSA, somatomedin A, NSILA-s, and insulin -- are biologically active in both chicken and human fibroblasts, stimulating thymidine incorporation into DNA. Our working hypothesis is that the induction of DNA synthesis by these peptides is mediated by their interaction with the growth peptide receptor.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47012-04 DB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Regulation of Receptors on Cultured Human Lymphocytes by Growth Hormone

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

PI:	M. A. Lesniak	Research Chemist	DB NIAMDD
	R. C. Eastman	Surgeon	DB NIAMDD
	J. Roth	Medical Director	DB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Diabetes Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	0.75	0.5

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

We had previously developed a radioreceptor assay to measure insulin and growth hormone using IM-9 cultured lymphocytes as the receptor and labeled polypeptide hormones as the ligand.

We have found that human growth hormone and cycloheximide alters the concentration of the growth hormone receptor on these cells. A decrease in receptor binding is dependent upon the concentration of the agents present as well as the duration of exposure of the agents to the cells. The decrease in receptor concentration is a reversible process and protein synthesis seems to be required for the restoration of receptors. The *in vitro* studies allows one to study the effects of agents upon the hormone specific receptors.

PHS-6040
(12-75)

Project No. Z01 AM 47,012-04 DB

Project Description:

Extending our previous work which showed that insulin is a major regulator of the insulin receptor, we now show the influence of hGH on the hGH receptor. When cultured human lymphocytes of the IM-9 line were preincubated with hGH at 37° for 18-24 hours, washed for 2 hours and then incubated with ^{125}I-hGH at 30° for 90 minutes, the binding of ^{125}I-hGH was reduced. The magnitude of the reduction of binding was dependent upon the concentration of hGH present as well as the duration of exposure of hGH to cells. For example, if hGH at 5.0 ng/ml (physiological concentration in man) was preincubated with the cells there is a 50% reduction in binding of ^{125}I-hGH. In the binding assay, this same concentration of hGH (5.0 ng/ml) occupied 20% of the receptor under steady state conditions. Further, 20 ng/ml hGH which occupied about 50% of the receptors in the binding assay produced an 80% loss of receptors when exposed to cells under preincubation conditions. Greater increases in growth hormone concentration produced little further effect on receptor loss. Analysis of the data indicated that a decrease in binding of ^{125}I-hGH was due to a loss of receptors per cell without any change in affinity of receptor for hormone or in cell density. The loss of receptor concentration due to preincubation of cells with hGH was a reversible process when the hGH is removed from the medium. Cycloheximide at 10^{-4}M inhibited the reversible process of receptor loss. However, when the cycloheximide was removed from the medium the receptor concentration returned to control level. The restoration of the receptors appeared to require the synthesis of new proteins. In IM-9 lymphocytes the concentration of growth hormone receptors is very sensitive to regulation by growth hormone.

Publications:

1. Roth, J: Assay of peptide hormones using cell receptors: Application to insulin and to human growth hormone. In Colowick, S. P. and Kaplan, N. O. (Eds.): Methods in Enzymology, Vol. 37. In O'Malley, B. W. and Hardmann, J. G. (Eds.): Peptide Hormones, New York, Academic Press, p. 66, 1975

2. Roth, J.: Methods for assessing immunologic and biologic properties of iodinated peptide hormones. In Colowick, S. P., and Kaplan, N. O. (Eds.) Methods in Enzymology, Vol. 37. In O'Malley, B. W., and Hardmann, J. G. (Eds.) Peptide Hormones, New York, Academic Press, pp. 223-233, 1975.

3. Roth, J., and Lesniak, M. A.: Regulation of human growth hormone (hGH) receptors by hGH: Studies with IM-9 lymphocytes. Proceedings of the 9th Miles Symposium, 1976, in press.

4. Lesniak, M. A., and Roth, J.: Regulation of receptor concentration by homologous hormone: Effect of human growth hormone on its receptor in IM-9 lymphocytes. J. Biol. Chem., 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47013-04 DB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Radioreceptor Assay of Growth Hormone Components

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

PI:	M. A. Lesniak	Research Chemist	DB	NIAMDD
	R. C. Eastman	Surgeon	DB	NIAMDD
	C. M. Hendricks	Bio Lab Tech	DB	NIAMDD
	P. Gorden	Senior Surgeon	DB	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Diabetes Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	0.75	1.5

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

The human growth hormone radioreceptor assay technique developed in this laboratory uses cultured human lymphocytes (IM-9 line) as the receptor and ^{125}I-hGH as the ligand. In conjunction with the radioimmunoassay the radioreceptor assay technique, proves to be a useful tool in which to measure not only the polypeptide hormone components but also the bioactivity of the hormone components in normal and disease states such as acromegaly. More studies involving other disease states are planned.

Project Description:

Many polypeptide hormones have been found to exist in glands and in plasma as multiple immunoreactive components which may have different biological activities. Growth hormone is no exception. Circulating and/or pituitary growth hormone when filtered on Sephadex has at least two discreet areas. We had designated these peak areas as "big" - ∿ 40,000 M.W. - and "little" - 22,000 M.W. - growth hormone. Both of these components have equal reactivity in the immunoassay. In previous experiments we had demonstrated that hGH can bind to specific sites on cultured human lymphocytes. Presently we have shown that these two discreet components have different radioimmuno - to radioreceptor activity. The ability of "big" hGH to compete for the binding sites is ∿ 20% less than "little" (monocomponent) hGH. Further studies have demonstrated that there are differences in the percent of receptor activity of the monomeric hGH in acromegalic patients when compared to normal subjects. This is true whether the data are expressed in only terms of "big" and "little" (89 vs 71%), as a function of total immunoreactive GH (76 vs. 55%) or whether the plasma is obtained in the basal or stimulated state for acromegalic patients.

Publications:

1. Gorden, P., Lesniak, M. A., and Eastman, R.: Evidence for higher proportion of "little" growth hormone with increased radioreceptor activity in acromegalic states. J. Endocrinol. Metab., 1976, in press.

2. Gorden, P., Lesniak, M. A., and Eastman, R.: Growth hormone receptors. Excerpta Medica, Holland, 1976, in press.

Annual Report of the Laboratory of Pediatric Metabolism Branch, National Institute of Arthritis, Metabolism and Digestive Diseases

During July 1, 1975 to June 30, 1976, investigations in cystic fibrosis of the pancreas have continued, with special emphasis on polyamines and vitamin E deficiency.

From our results, as well as a critical evaluation of those in the literature, it seems improbable that the basic defect in CF consists of the absence of a single enzyme in a major metabolic pathway with a build-up of precursors and a lack of products, or a primary flaw in glycoprotein or glycosaminoglycan metabolism. It must account for a generalized exocrinopathy, but with morphologic and physiologic normality of the exocrine glands prior to the onset of the pathologic effects of disease. Homozygotes with CF would do well, and lead essentially normal lives, were it not for the secondary changes due to illness (i.e., chronic pulmonary disease, pancreatic deficiency, etc.) The basic defect in CF, therefore, may be a quantitative rather than a qualitative difference, perhaps regulatory in nature.

Control of the secretory process by the autonomic nervous system or by other factors is a possible site of the CF defect and any step in this linkage, from the receptor to the cyclic AMP, cyclic GMP or intracellular calcium, is suspect. The pathologic and physiologic role of polyamines was also subject to continued investigation. However, although CF research is "so near and yet so far" from uncovering the basic error, investigations into the origins of this common, clinically well-described and easily recognized illness have already yielded, and will continue to yield, important dividends in basic understanding of the secretory process, polyamine physiology, and the biochemistry of small serum proteins. Along more practical lines, from studies of patients with CF, important nutritional information has been obtained as to the effects in man of vitamin E deficiency and, conversely, as to the needlessness of supplementation of this vitamin in normal individuals in advanced countries.

I. Biochemical and Metabolic Studies

A. Investigations on Polyamines

It has been previously shown in this laboratory (Clinica Chimica Acta 62:357, 1975) that male homo- and heterozygotes for CF exhibit a consistent and significant decrease in whole blood spermine resulting in an elevated control spermidine-to-spermine ratio, when compared to control males. The exact significance of this abnormality is obscure, and whether it is primary or secondary to the as-yet-unknown basic defect remains speculative. Its presence in heterozygotes strongly suggests that this is a gene-related phenomenon and not a result of the disease. While the physiology of polyamines in man is still being elucidated, certainly processes which may be abnormal in CF, such as glycosyl-transferase activity and salt transport, are known to be influenced by polyamines in other systems. Anyway, they are the first well-characterized metabolite of low molecular weight to be demonstrated to be abnormal in CF.

1. Influence of the menstrual cycle on blood polyamine levels

Close inspection of the polyamine levels and spermidine/spermine ratios from female controls and female homo- and heterozygotes for CF suggest that they are subject to greater variability than male values. As this is not consistent with the accepted mode of inheritance of this genetic disease (autosomal recessive), this observation suggested that a sex-related hormone(s) might be influencing the female spermidine-to-spermine (Spd/Spm) ratio during the menstrual cycle.

Accordingly, percholoric acid-extractable whole blood spermidine and spermine concentrations were determined over a four-week period in three men, four normal women, and one ovariectomized woman, as well as the progesterone and estradiol levels in the females. Individual male Spd/Spm ratios showed little fluctuation and similar values were obtained for each of the three males studied, although the actual concentrations of both compounds varied from one subject to the next. Individual female Spd/Spm ratios, as well as individual concentrations, fluctuate substantially when compared with the values obtained for males, and appeared to rise and fall as a function of the menstrual cycle. The Spd/Spm ratios obtained from a normal female receiving oral contraceptives, as well as those from an ovariectomized female, were characteristic of the values obtained from men.

It was concluded that a sex-related hormone(s) influences both the Spd/Spm ratio and the actual spermidine and spermine concentrations in females. This observation may have great significance for oncologists, as blood and urinary polyamine levels have been shown to rise with tumor growth and fall with tumor regression (Dr. D. Lundgren, Dr. P. Farrell, Dr. L. Cohen, NIAMDD).

2. Localization of spermidine and spermine in formed elements of blood

Studies were also continued in this fiscal year on the localization of these two polyamines in formed elements of blood of 13 male CF patients and 14 healthy male controls. To investigate Spd and Spm distribution among blood components as a possible cause of the abnormality, blood was fractionated using Rabinowitz's glass bead technique and Boyum's ficoll-Hypaque method. Polyamines were extracted with percholoric acid and quantitated on an amino acid analyzer. In controls, mean ± SEM concentration in nmoles per 10^9 cells of Spd and Spm, respectively, were 1.02 ± .08 and .894 ± .28 for erythrocytes; 126 ± 31 and 357 ± 105 for lymphocytes; 36 ± 16 and 240 ± 33 for granulocytes; and <0.5 and <0.5 nmoles/ml for plasma. When converted to the concentration in whole blood, it was found that greater than 90% of Spd and over 70% of Spm was associated with erythrocytes.

While the higher cellular concentration in leukocytes is not unexpected, the fact that Spd and Spm in whole blood are primarily associated with erythrocytes is a new finding. Comparison with controls revealed that Spd/Spm ratio in both whole blood and erythrocytes were significantly higher in the group of CF patients. (Dr. L. Cohen, Dr. D. Lundgren, Dr. P. Farrell, Dr. P di Sant' Agnese, NIAMDD).

3. Polyamine uptake by human erythrocytes

Previous studies in this laboratory have shown that 80-90% of the Spd and Spm found in human whole blood are localized in erythrocytes and that individuals with cystic fibrosis have an increased Spd/Spm ratio in their RBC's when compared to control subjects. The former observation is surprising in light of the numerous reports implicating the polyamines as important metabolites in growth and development and their association with DNA and RNA. On the other hand, erythrocytes might play an important role in polyamine metabolism by: 1) transporting polyamines to more proliferating tissues, 2) maintaining extracellular steady state concentrations, or 3) being a site of polyamine detoxification, since polyamines and their catabolic products have been shown, under certain conditions, to be toxic to various mammalian systems.

In an attempt to understand the physiological relationship between RBC's and polyamines, in vitro studies have been initiated: 1) to determine if polyamines are taken up by RBC's and, if so, 2) to characterize the uptake, and 3) to compare uptake in cystic fibrosis and normal RBC's.

Putrescine, spermidine and spermine are taken up by RBC's and uptake is proportional to incubation time as well as RBC concentrations. At 1 mM concentrations, putrescine (Put) is taken up to a much greater extent than Spd, and Spd to a greater degree than Spm. Uptake is temperature-dependent and pH-dependent. As the pH is increased from 7.4 to 10.0,and the polyamines are neutralized, uptake is markedly enhanced. Uptake as a function of substrate concentration produced hyperbolic kinetics for all three cations and each system was saturated at approximately 50 mM Put, Spd or Spm. Neither arginine (Arg) nor lysine (Lys), 1 mM, inhibited polyamine uptake. Ouabain (1 mM) and Na F (.1 mM) did not inhibit polyamine uptake, but N-ethylmalimide (3 mM) inhibited Put uptake by 30%.

Of interest is the observation that the addition of serum to the incubation mixture, in place of an equal amount of incubation medium, enhanced Put uptake by as much as 200%, Spd uptake by 70% and Spm uptake by 30%, whereas the uptake of Arg or Lys is not enhanced by the addition of serum. Investigation to determine the nature of the increased uptake produced by serum is presently being made. (Dr. D. Lundgren, NIAMDD, C. Jay, Guest Worker, NIAMDD).

4. Electrolyte and polyamine values in sweat from cystic fibrosis patients and normal subjects

Previous studies in this laboratory have shown that the spermidine/spermine ratio is elevated in perchloric acid-extracted whole blood from CF patients when compared to control subjects. Since the only consistently demonstrable abnormality characteristic of individuals with CF is elevated sweat sodium and chloride, and polyamines have been reported to be involved in maintaining appropriate salt balance across cellular membranes in *E. coli*, studies have been initiated to determine putrescine, spermidine and spermine concentrations in CF patients' and control subjects' sweat.

For this purpose, whole body sweat was collected in approximately 50 ml volumes using a metabolic chamber (120 F, 10-12% relative humidity). Sweat from four normal volunteers and three CF patients has been analyzed to date and the results are provided in the following table:

CONCENTRATIONS OF SELECTED AMINES* IN WHOLE BODY SWEAT FROM MALES

	Concentration (nmoles/ml) Arginine	Putrescine	Ratio Avg/Put
Normal controls	12.6	4.91	2.56
	25.0	6.98	3.58
	15.0	9.21	1.63
	16.7	6.56	2.55
Mean ± SEM	17.3 ± 2.7	6.91 ± 0.89	2.58 ± 0.4
Cystic fibrosis	21.3	5.34	3.99
	18.6	4.36	4.27
	19.6	5.15	3.80
Mean ± SEM	19.8 ± 0.79	4.95 ± 0.3	4.02 ± 0.14**

*Spermidine and spermine were undetectable in concentrated sweat samples (sensitivity of this method is 0.5 nmoles/ml).

**$p < 0.05$, as compared to the arginine/putrescine ratio in the control group.

From the values listed, it is evident that Put and Arg, a metabolic precursor to polyamine biosynthesis, are present in both CF and control sweat; neither Spd nor Spm could be detected. The number of samples evaluated in this initial series is too small to permit conclusions; however, there is a suggestion that Arg levels may be high and Put levels low in CF sweat. The ratio of Arg/Put is significantly increased (<0.5) in this group of CF patients, as compared to controls.

Although localized thermal stimulation has been utilized in some laboratories to determine the sodium and chloride levels in individuals suspected of having cystic fibrosis, the method in general use at present for the diagnosis of this disorder is localized pilocarpine iontophoresis. Electrolyte concentrations in whole body sweat have been compared to samples collected from small areas of skin surface in normal subjects, but never, to our knowledge, in CF patients. Such information would be useful for a number of reasons, such as the feasibility of collecting large volumes of sweat to be utilized in the isolation of factors reported to be in CF sweat as well as for the determination of polyamine levels in CF vs. normal sweat, in which case large volumes of sweat must be obtained and concentrated before these cations can be quantitated.

Accordingly, whole body sweat was collected via a metabolic chamber for polyamine determinations, to ascertain if electrolyte concentrations and sweat rates are comparable to the values in sweat obtained via pilocarpine iontophoresis in both normal and pathologic subjects. As can be seen from the following table, the electrolyte composition from whole body sweat is quite similar to the values obtained for both CF and control subjects. The whole body sweat rate, however, is approximately twice the value obtained from the arm patch method. When sodium rate-sweat slopes (not shown) obtained in this study for whole body sweat are compared to those obtained by others for small sweat samples from CF patients, an excellent correlation was found to exist.

ELECTROLYTE COMPOSITION AND SECRETION RATES OF SWEAT SAMPLES

COLLECTED FROM LOCAL AND WHOLE BODY SKIN SURFACES*

Concentration or secretion rate	Control (n=5)		Cystic Fibrosis (n=18)	
	Whole Body	Arm Patch	Whole Body	Arm Patch
Sodium (mEq/l)	37.0 ± 11	34.0 ± 14	103.5 ± 19**	107.4 ± 17**
Chloride (mEq/l)	24.6 ± 12	23.6 ± 11	103.8 ± 16**	105.2 ± 17**
Sweat rate ($gm/min/m^2$)	3.52 ± 0.96	2.80 ± 0.27	4.13 ± 1.2	2.24 ± .91†
Na excretion rate ($mEq/min/m^2$)	0.124 ± 0.03	0.094 ± 0.03	0.450 ± .14**	0.244 ± .11**†

*Mean ± SD figures are listed and were analyzed with the Student t test.

**p <.001 vs. control value.
†p <.001 vs. WBS.

In conclusion, the similar sodium rate-sweat slopes (not shown) and the excellent correlation obtained in these studies between the electrolyte concentrations indicate that sodium and chloride levels in the final sweat product are independent of the collection method utilized. Therefore, the 1000-fold larger quantities of sweat obtained from whole body surfaces by thermal stimulation are comparable to the small amounts collected locally after routine pilocarpine iontophoresis. Thus, whole body sweat may be useful for further chemical analysis and comparison of CF to normal sweat, such as polyamine concentrations. (Dr. P. Farrell, Dr. D. Lundgren, Dr. L.Cohen, Dr. P.di Sant'Agnese, NIAMDD).

B. Fucose Incorporation by Fibroblasts

Because of the sweat electrolyte defect and a presumed anomaly in the exocrine secretion process, the plasma membranes of CF cells have been examined by several workers. One group reported that CF serum lacked a material present in normal serum which stimulated incorporation of ^{3}H-L-fucose into normal or CF fibroblast plasma membranes (Chou and Nadler, Ped Res 9:312, 1975). We attempted to reproduce these experiments, but found that CF and normal serum both stimulated fucose incorporation by fibroblasts to the same extent, under a wide variety of conditions of handling of serum, fibroblast growth, harvesting and processing of samples. No differences were found in fucose incorporation by CF fibroblasts, compared to normals, when results were expressed per 10^{6} cells; however, since CF fibroblasts contain only 2/3 the protein of normal cells, incorporation of ^{3}H-L-fucose per mgm fibroblast protein was 1.5 times as great in CF cells as normals. Thus, we are unable to confirm the observations of Chou and Nadler with respect to ^{3}H-L-fucose incorporation by fibroblasts. (Dr. P. Davis, H.S. Chambers, NIAMDD).

C. Cyclic Nucleotides and CF

The role of the cyclic nucleotides in exocrine secretory processes is still being determined, but it is suspected that they mediate the influence of the autonomic nervous system on the glands and may be essential determinants of secretion. These compounds have received relatively little attention in cystic fibrosis, a disorder of exocrine secretion. We have taken several approaches to the role of cyclic nucleotides in cystic fibrosis.

1. Cyclic AMP and cyclic GMP in submaxillary saliva and urine

Four CF patients and three normal controls of comparable age underwent collection of submaxillary saliva in the fasting state with the aid of a Schneyer segregator. Submaxillary saliva was selected because this predominantly mucous secretion has been shown to be abnormal in CF. Preliminary results indicate that there is little difference in concentration of cAMP and cGMP between normal and CF submaxillary saliva. See chart below:

		Saliva	
		cAMP (pmole/ml)	cGMP (pmole/ml)
Normal	N=5	16.2 ± 3.0	1.73 ± .35
CF	N=6	19.8 ± 7.0	1.50 ± .24

However, because of the large variation in CF cAMP samples, more patients may be studied, and parotid saliva (predominantly serous secretion) may be collected by the Lashley cup technique and assayed.

Murad et al. have reported abnormalities in cAMP and cGMP excretion in CF, compared to age-matched controls (J Endocr & Metab 10:552, 1975). We have determined cAMP and cGMP in 24-hour urine from adult male CF patients and appropriate controls and have found that older CF patients have slightly increased urinary cAMP, but markedly elevated urinary cGMP. See chart below:

	Urine	
	cAMP (nmole/g creat)	cGMP (nmole/g creat)
Normal N=5	2.23 ± 0.4	0.16 ± 0.01
CF N=6	2.88 ± 0.3	0.28 ± 0.09

Moreover, we are investigating the relation of urinary cAMP and cGMP to the degree of illness as gauged by the NIH Clinical Score. This study is continuing as more data is required for statistically valid conclusions, and we plan to extend the study to heterozygotes, to determine whether these changes are gene related. (Dr. R. Wolf, NIDR, Dr. P. Davis, H.S. Chambers, NIAMDD).

2. Isoproterenol stimulation of cyclic AMP in lymphocytes and fibroblasts

Canadian workers have shown that CF fibroblasts respond with 3-15 times more cAMP to isoproterenol stimulation than do normal cells. We are currently attempting to study the β receptor and its cAMP response in fresh leukocytes and cultured fibroblasts. The leukocyte assay must first be standardized with respect to the state of the subject from whom the blood is obtained, and we are currently exploring the relation of feeding, exercise and the menstrual cycle to these assays. (Dr. P. Davis, M. Braunstein, NIAMDD, Dr. J. Tallman, NIMH).

D. Vitamin E in Man

Studies of vitamin E deficiency in man and on the effects in humans of megavitamin E supplementation have been completed and are giving rise to numerous publications.

These investigations have been very productive and it has been shown conclusively that vitamin E deficiency in man, using patients with CF as models (as they have profound and long-standing deficiency of this supplement) produces in humans many of the features seen in experimental animals, although usually at a sub-clinical level. Among others, erythrocyte hemolysis is increased, erythrocyte survival is shortened and there are indications of muscle involvement, as shown by creatinuria and, at times, by increases in plasma creatinine-kinase and aldolase. Conversely, on electron microscopy, there are no morphologic changes visible in subcellular organelles in

intestinal biopsies. Most of the pathologic findings are corrected, in vitro and in vivo, by administration of vitamin E. On the other hand, normal controls had normal levels of tocopherol in plasma, regardless of vitamin E supplementation. It was concluded that patients with CF and pancreatic deficiency, and other malabsorptive causes of vitamin E deficiency, as well as populations in areas of the world exposed to famine conditions (e.g., Bangladesh, Jordan, Ethiopia, etc.) and receiving inadequate diets, should be supplemented with vitamin E.

In contrast, a study of normal adults voluntarily taking large amounts of this vitamin did not show any benefits from the supplementation, despite significantly higher levels of plasma α-tocopherol. No toxicity effects were noted, probably because the α-tocopherol levels correlate with plasma triglyceride and cholesterol concentrations, and therefore do not increase beyond a certain level, regardless of the amount of vitamin E ingested. There were no specific beneficial effects which were noted consistently. It was concluded that vitamin E supplementation is not necessary or advisable in normal individuals in this and other advanced countries, in which the diet is adequate. (Dr. P. Farrell, Dr. J. Bieri, Dr. P. di Sant'Agnese, J. Willison, NIAMDD, Dr. V. Fischer, University of St. Louis, Mo.).

II. Clinical Studies

A. Adults with Cystic Fibrosis

CF was long thought to be the exclusive province of the pediatrician, since in the pre-antibiotic era survival into adolescence was rare. In addition, the patients in the older age group, who frequently do not present the textbook picture of this disease, were rarely diagnosed. Frequently, internists have seen little of this disease during their training and do not recognize it. In this Branch, we have specialized in older patients with CF for several different reasons relating to investigations, and also because they needed better clinical, diagnostic and therapeutic definition.

We are now analyzing the cases of 62 adults with CF over 18 years of age, the oldest being 46. While the clinical picture as a whole is similar to that of children with this disorder, there are many points of difference. Many of the older patients are quite tall (several are over 6 ft.) and well-nourished, regardless of how late the diagnosis was made, and therefore even if they had no treatment, or certainly not optimal care. The question was thus raised, and is supported by other findings. of the possibility of a different genetic type of the CF syndrome in which patients live longer, thus raising the question as to the existence of more than one type of this disease, although masquerading under the same symptom complex.

Some of the complications which occur in older patients with cystic fibrosis are either not seen at all or are rarely seen in children: pneumothorax, massive hemoptysis, ileo-cecal intussusception, fecal masses in the cecum leading to obstruction, glucose intolerance with glycosuria frequently mistaken for "genetic" diabetes mellitus, sinusitis and nasal polyposis, aspermia in males and reduced fertility in females. From the diagnostic

standpoint, interpretation of the sweat test, still the cardinal confirmatory test, should be more cautious, because sweat electrolytes tend to increase somewhat with age in normal individuals. The other diagnostic criteria (pancreatic deficiency and chronic pulmonary disease) in general should be required, as well as a sputum revealing either Staph aureus or Pseudomonas aeruginosa with mucoid degeneration, the latter frequently as the only or predominant organism present in the respiratory tract. The type of treatment for older patients with CF is somewhat different from that of children, as they seem to need less dietary restrictions, while, conversely, other features, such as cardiac failure and the syndrome of inappropriate antidiuretic hormone secretion, seem to be more common. It should be noted that older CF patients may tolerate chronic Pseudomonas infection relatively well, as they have gradually built up immunological defenses against this organism elsewhere in the body. This is in contrast to the usual fulminant course of Pseudomonas infections, in immunosuppressed or burned patients.

B. Survey of Pregnancy in CF

In dealing with females with CF who needed antibiotic treatment during pregnancy, it was found that almost nothing was available on the subject in the literature as guidance. We have, therefore, been conducting a survey throughout the United States, and have found 129 pregnancies in 100 females with cystic fibrosis. There were 10 maternal deaths within six months of term and an additional five from 6 to 24 months post partum. In 94% of the patients on whom information was obtained, antibiotics, frequently quite toxic, had to be used during pregnancy to permit survival of the mother and child. In general, only patients who were relatively well had become pregnant, so that there were only 10 maternal deaths within 6 months of term. There were 70 full-term infants, 16 pre-term, 30 abortions (most spontaneous) and 11 cases of either stillbirth or neonatal death within 48 hours. Only one child of the 70 had CF: the expected incidence when a homozygote female mates with a male of unknown genetic status.

Normally, there is increased respiratory and cardiac work, especially hypervolemia, during pregnancy. CF patients develop early cor pulmonale and it is known that obstructive pulmonary diseases are more severely affected than the restrictive ones during pregnancy. Therefore, it was felt that pregnancy in CF is hazardous for the mother and fetus, and not advisable unless the lung disease is mild (NIH Score >80). Therapeutic abortion was felt to be indicated if a significant degree of pulmonary involvement was present. Last, antibiotic treatment, even with anti-Pseudomonas agents, is absolutely necessary during pregnancy despite possible hazards, in order to save the mother and offspring. (Dr. L. Cohen, Dr. P. di Sant'Agnese, J. Friedlander, NIAMDD).

C. Syndrome of Inappropriate Antidiuretic Hormone Secretion in CF and in Pediatrics (SIADH)

SIADH was observed in two patients with CF, during acute exacerbation of chronic pulmonary disease. It was diagnosed by clinical and laboratory criteria, and confirmed by values for immunoreactive vasopressin inappropriately

high for plasma osmolality. The marked hyponatremia and unusually severe clinical symptoms responded to intensive treatment.

SIADH may be more common than is realized in CF and there are several past patients with long-standing and advanced pulmonary disease who had severe hyponatremia, never clearly explained, and usually as a terminal phenomenon. It is especially important to recognize SIADH promptly in CF, as the treatment is the exact opposite for that of hyponatremia due to salt loss because of sweating (fluid restriction, rather than administration). As both circumstances may represent an acute medical emergency, prompt and correct diagnosis may have fateful therapeutic consequences.

This in general is true in pediatrics, as most other causes of marked or profuse hyponatremia in this age group are associated with dehydration: again, requiring administration of fluid and electrolytes, rather than fluid restriction. The pediatric literature is singularly deficient in reporting cases of SIADH, although a consideration of the subject leads us to the conclusion that further search in this age group may reveal many unrecognized cases, with important and frequently emergent therapeutic implications, especially as in this age group (pneumonia, meningitis, etc.) prompt and proper treatment of hyponatremia may afford the margin of safety until effective antibacterial and other therapy can take effect. (Dr. L. Cohen, Dr. P. di Sant'Agnese, NIAMDD).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 49000-03-PMB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Polyamine Biochemistry and Physiology and Their Relationship to Cystic Fibrosis

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

PI:	D.W. Lundgren	Staff Fellow, Pediatric Metabolism Branch	PMB:NIAMDD
	L.F. Cohen	Clinical Assoc., Pediatric Metabolism Branch	PMB:NIAMDD
OTHERS:	P.M. Farrell	Sr. Invest., Pediatric Metabolism Branch	PMB:NIAMDD
	P.A. di Sant'Agnese	Chief, Pediatric Metabolism Branch	PMB:NIAMDD

COOPERATING UNITS (if any)

none

LAB/BRANCH
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SECTION

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NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.8	.5

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

The polyamines, spermidine (Spd) and spermine (Spm) have been measured in whole blood extracts from control volunteers, patients with cystic fibrosis(CF), and CF obligate heterozygotes. Male homo- and heterozygotes for CF exhibit an elevated Spd/Spm ratio. Female Spd/Spm ratios fluctuate substantially when compared with values obtained for males, and appear to rise and fall as a function of the menstrual cycle. Whole blood fractionated into its various cellular components revealed that greater than 90% of Spd and 70% of Spm were associated with erythrocytes. Comparison with control subjects revealed that Spd/Spm ratios in erythrocytes were significantly higher in CF patients. Spd and Spm were not found in whole body sweat although putrescine was found to be present. Preliminary data suggest that the arginine/putrescine ratio in sweat of individuals may be increased when compared to values obtained from controls. Comparison of electrolyte concentrations and sweat rates via a metabolic chamber to values obtained via pilocarpine iontophoresis were found to be comparable in both normal and pathologic subjects.

Objectives: The physiological role of the diamine, putrescine, and the polyamines spermidine and spermine is not known. It has been previously shown in this laboratory that male homo- and heterozygotes for CF exhibit a consistent and significantly elevated spermidine to spermine ratio, when compared to control males. The exact significance of this abnormality is obscure, and whether it is primary or secondary to the as-yet-unknown basic defect remains speculative. The observation of these differences in a) homozygotes and b) clinically normal obligate heterozygote fathers suggested the importance of followup investigations. Therefore, studies have been initiated to afford a better understanding of the physiological role of polyamines and their potential relationships to cystic fibrosis.

Methods: The following procedures were performed: extraction of whole blood polyamines and analysis via an amino acid analyser {^{14}C};polyamine uptake in human erythrocytes; blood fractionation via glass bead chromatography and ficoll-Hypaque; collection of whole body sweat using a metabolic chamber.

Major Findings:

1) Influence of the menstrual cycle on blood polyamine levels:

Whole blood polyamine levels and spermidine/spermine ratios were found to be abnormal in male,but not female, homo- and heterozygotes. As this is not consistent with the accepted mode of inheritance of this genetic disease (autosomal recessive), this observation suggested that a sex-related hormone(s) may influence the female spermidine to spermine (Spd/Spm) ratio during the menstrual cycle.

Accordingly, perchloric acid-extractable whole blood spermidine and spermine concentrations were determined over a four-week period in three men, four normal women and one ovariectomized woman; progesterone and estradiol levels were determined in the females. Individual male Spd/Spm ratios showed little fluctuation and similar values were obtained for each of the three males studied, although the actual concentrations of both compounds varied from one subject to the next. Individual female Spd/Spm ratios, as well as individual concentrations, fluctuate substantially when compared with the values obtained for males, and appeared to rise and fall as a function of the menstrual cycle. The Spd/Spm ratios obtained from a normal female receiving oral contraceptives, as well as those from an ovariectomized female, were characteristic of values obtained from men.

It was concluded that a sex-related hormone(s) influences both the Spd/Spm ratio and the actual spermidine and spermine concentrations in females. This observation may have great significance for oncologists, as blood and urinary polyamine levels have been shown to rise with tumor growth and fall with tumor regression, and are used experimentally to monitor response to therapy.

2) Localization of spermidine and spermine in formed elements of blood:

To investigate Spd and Spm distribution among blood components as a possible cause of the abnormality, blood was fractionated using Rabinowitz's glass bead technique and Boyum's ficoll-Hypaque method. Polyamines were extracted with perchloric acid and quantitated on an amino acid analyser. In controls, mean ± SEM concentration in nmoles per 10^9 cells of Spd and Spm, respectively, were 1.02 ± .08 and .894 ± .28 for erythrocytes; 126 ± 31 and 357 ± 105 for lymphocytes; 36 ± 16 and 240 ± 33 for granulocytes; and < 0.5 and < 0.5 nmoles/ml for plasma. When converted to the concentration in whole blood, it was found that greater than 90% of Spd and over 70% of Spm was associated with erythrocytes.

While the higher cellular concentration in leukocytes is not unexpected, the fact that Spd and Spm in whole blood are primarily associated with erythrocytes is a new finding. Comparison with controls revealed that Spd/Spm ratio in both whole blood and erythrocytes were significantly higher in the group of CF patients.

In an attempt to understand the physiological relationship between RBC's and polyamines, in vitro studies have been initiated: 1) to determine if polyamines taken up by RBC's and, if so, 2) to characterize the uptake, and 3) to compare uptake in cystic fibrosis and normal RBC's.

Putrescine, spermidine and spermine are taken up by RBC's and uptake is proportional to incubation time as well as RBC concentrations. At 1 mM concentrations, putrescine (Put) is taken up to a much greater extent than Spd, and Spd to a greater extent than Spm. Uptake is temperature-dependent and pH-dependent. As the pH is increased from 7.4 to 10.0 and the polyamines are neutralized, uptake is markedly enhanced. Uptake as a function of substrate concentration produced hyperbolic kinetics for all three cations and each system was saturated at approximately 50 mM Put, Spd or Spm. Neither arginine (Arg) nor lysine (Lys), 1 mM, inhibited polyamine uptake. Ouabain, 1 mM, and Na F, .1 mM, did not inhibit polyamine uptake, but N-ethylmalimide, 3 mM, inhibited Put uptake by 30%.

Of interest is the observation that the addition of serum to the incubation mixture, in place of an equal amount of incubation medium, enhanced Put uptake by as much as 200%, Spd uptake by 70% and Spm uptake by 30%, whereas the uptake of Arg or Lys is not enhanced by the addition of serum. Investigation to determine the nature of the increased uptake produced by serum is in progress.

3) Electrolyte and polyamine values in sweat from cystic fibrosis patients and normal subjects:

Since the only consistently demonstrable abnormality characteristic of individuals with CF is elevated sweat sodium and chloride, and polyamines have been reported to be involved in maintaining appropriate salt balance across cellular membranes in E. coli, studies have been initiated to determine putrescine, spermidine and spermine concentrations in CF and control sweat.

For this purpose, whole body sweat was collected in approximately 380-860 ml volumes using a metabolic chamber (120 F, 10-12% relative humidity). Sweat from four normal volunteers and three CF patients has been analysed to date and the results are provided in the following table:

CONCENTRATIONS OF SELECTED AMINES* IN WHOLE BODY SWEAT FROM MALES

	Concentration (nmoles/ml) Arginine	Putrescine	Ratio Avg/Put
Normal controls:	12.6	4.91	2.56
	25.0	6.98	3.58
	15.0	9.21	1.63
	16.7	6.56	2.55
Mean ± SEM	17.3±2.7	6.91±0.89	2.58±0.4
Cystic fibrosis:	21.3	5.34	3.99
	18.6	4.36	4.27
	19.6	5.15	3.80
Mean ± SEM	19.8±0.79	4.95±0.3	4.02±0.14**

*Spermidine and spermine were undetectable in concentrated sweat samples (sensitivity of this method is 0.5 nmoles/ml).

**p <0.05 as compared to the arginine/putrescine ratio in the control group.

From the values listed, it is evident that Put and Arg, a metabolic precursor to polyamine biosynthesis, are present in both CF and control sweat; neither Spd nor Spm could be detected. The number of samples evaluated in this initial series is too small to permit conclusions; however, there is a suggestion that Arg levels may be high and Put levels low in CF sweat. The ratio of Arg/Put is significantly increased ($p < .05$) in this group of CF patients, as compared to controls.

Although localized thermal stimulation has been utilized in some laboratories to determine the sodium and chloride levels in individuals suspected of having cystic fibrosis, the method in general use at present for the diagnosis of this disorder is localized pilocarpine iontophoresis. Electrolyte concentrations in whole body sweat have been compared to samples collected from small areas of skin surface in normal subjects, but never, to our knowledge, in CF patients. Such information would be useful for a number of reasons, such as the feasibility of collecting large volumes of sweat to be

utilized in the isolation of factors reported to be in CF sweat as well as for the determination of polyamine levels in CF vs. normal sweat, in which case large volumes of sweat must be obtained and concentrated before these cations can be quantitated.

Accordingly, whole body sweat was collected via a metabolic chamber for polyamine determinations to ascertain if electrolyte concentrations and sweat rates are comparable to the values in sweat obtained via pilocarpine iontophoresis in both normal and pathologic subjects.

A high level of correlation for the concentrations of Na (r=.857) and Cl (.893) was found for the two methods. As can be seen from the following table, the mean electrolyte composition from whole body sweat is quite similar to the values obtained by local methods for both CF and control subjects. The whole body sweat rate, however, is approximately twice the value obtained from the arm patch method. When sodium excretion rate-sweat rate slopes (not shown) obtained by each method are compared, an excellent correlation is found for CF patients.

ELECTROLYTE COMPOSITION AND SECRETION RATES OF SWEAT SAMPLES

COLLECTED FROM LOCAL AND WHOLE BODY SKIN SURFACES*

Concentration or secretion rate	Control (n=5)		Cystic Fibrosis (n=18)	
	Whole Body	Arm Patch	Whole Body	Arm Patch
Sodium (mEq/l)	37.0 ± 11	34.0 ± 14	103.5 ± 19**	107.4 ± 17**
Chloride (mEq/l)	24.6 ± 12	23.6 ± 11	103.8 ± 16**	105.2 ± 17**
Sweat rate ($gm/min/m^2$)	3.52 ± 0.96	2.80 ± 0.27	4.13 ± 1.2	2.24 ± .91†
Na excretion rate ($mEq/min/m^2$)	0.124 ± 0.03	0.094 ± 0.03	.450 ± .14**	.244 ± .11**†

*Mean ± SD figures are listed and were analysed with the Student t test.

** p <.001 vs. control value.

† p <.001 vs. WBS.

In conclusion, the similar sodium excretion rate-sweat rate slopes (not shown) and the excellent correlation obtained in these studies between the electrolyte concentrations indicate that sodium and chloride levels in the final sweat product are independent of the collection method utilized.

Therefore, the 1000-fold larger quantities of sweat obtained from whole body surfaces by thermal stimulation are comparable to the small amounts collected locally after routine pilocarpine iontophoresis. Thus, whole body sweat may be useful for further chemical analysis and comparison of CF to normal sweat, such as polyamine concentrations.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 49001-03-PMB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Clinical Studies in Cystic Fibrosis

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

PI:	P.A. di Sant'Agnese	Chief, Pediatric Metabolism Branch	PMB:NIAMDD
OTHERS:	L.F. Cohen	Clinical Assoc., Pediatric Metabolism Branch	PMB:NIAMDD
	P.B. Davis	Clinical Assoc., Pediatric Metabolism Branch	PMB:NIAMDD
	P.M. Farrell	Sr. Invest., Pediatric Metabolism Branch	PMB:NIAMDD

COOPERATING UNITS (if any)

LNE:NIAMDD (J. Bieri, Chief, Nutritional Biochemistry Section)

LAB/BRANCH

Pediatric Metabolism Branch

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	5	1

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

It is the long-range goal of this project to study the manifestations of cystic fibrosis with particular reference to the adult age group. We are collating our experience with over 60 adult patients; we have analyzed data regarding pregnancy in cystic fibrosis provided by cooperating CF centers across the country; and we have described clinical syndromes (SIADH) not previously reported in CF. Also, we have studied metabolism and clinical significance of vitamin E in normal and cystic fibrosis subjects.

PHS-6040
(12-75)

Objectives: To clarify many clinical features of cystic fibrosis, with special reference to adults, and to improve treatment of this condition.

1) Adults with cystic fibrosis.

CF was long thought to be the exclusive province of the pediatrician, since in the pre-antibiotic era survival into adolescence was rare. In addition, the patients in the older age group, who frequently do not present the textbook picture of this disease, were rarely diagnosed. Frequently, internists have seen little of this disease during their training and do not recognize it. In this Branch, we have specialized in older patients with CF for several different reasons relating to investigations, and also because they needed better clinical, diagnostic and therapeutic definition.

We are now analyzing the cases of 62 adults with CF over 18 years of age, the oldest being 46. While the clinical picture as a whole is similar to that of children with this disorder, there are many points of difference. Many of the older patients are quite tall (several are over 6 ft.) and well-nourished, regardless of how late the diagnosis was made, and therefore even if they had no treatment, or certainly not optimal care. The question was thus raised, and is supported by other findings, of the possibility of a different genetic type of the CF syndrome in which the patients live longer, thus raising the question of the existence of more than one type of this disease, although masquerading under the same symptom complex.

Some of the complications which occur in older patients with cystic fibrosis are either not seen at all or are rarely seen in children: pneumothorax, massive hemoptysis, ileo-cecal intussusception, fecal masses in the cecum leading to obstruction, glucose intolerance with glycosuria frequently mistaken for "genetic" diabetes mellitus, sinusitis and nasal polyposis, aspermia in males and reduced fertility in females. From the diagnostic standpoint, interpretation of the sweat test, still the cardinal confirmatory test, should be more cautious, because sweat electrolytes tend to increase somewhat with age in normal individuals. The other diagnostic criteria (pancreatic deficiency and chronic pulmonary disease) in general should be required, as well as a sputum revealing either Staph aureus or Pseudomonas aeruginosa with mucoid degeneration, the latter frequently as the predominant or only organism present in the respiratory tract. The type of treatment for older patients with CF is somewhat different from that of children, as they seem to need less dietary restrictions, while, conversely, other features, such as cardiac failure and the syndrome of inappropriate antidiuretic hormone secretion, seem to be more common. It should be noted that older CF patients may tolerate chronic Pseudomonas infection relatively well, as they have gradually built up immunological defenses against this organism elsewhere in the body. This is in contrast to the usual fulminant course of Pseudomonas infections, in immunosuppressed or burned patients.

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Normally, there is increased respiratory and cardiac work, especially hypervolemia, during pregnancy. CF patients develop early cor pulmonale and it is known that obstructive pulmonary diseases are more severely affected that the restrictive ones during pregnancy. Therefore, it was felt that pregnancy in CF is hazardous for the mother and fetus, and not advisable unless the lung disease is mild (NIH Score >80). Therapeutic abortion was felt to be indicated if a significant degree of pulmonary involvement was present. Last, antibiotic treatment, even with anti-Pseudomonas agents, is absolutely necessary during pregnancy despite possible hazards, in order to save the mother and offspring.

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SIADH was observed in two patients with CF, during acute exacerbation of chronic pulmonary disease. It was diagnosed by clinical and laboratory criteria, and confirmed by values for immunoreactive vasopressin inappropriately high for plasma osmolality. The marked hyponatremia and unusually severe clinical symptoms responded to intensive treatment.

SIADH may be more common than is realized in CF and there are several past patients with long-standing and advanced pulmonary disease who had severe hyponatremia, never clearly explained, and usually as a terminal phenomenon. It is especially important to recognize SIADH promptly in CF, as the treatment is the exact opposite for that of the hyponatremia due to salt loss because of sweating (fluid restriction, rather than administration). As both circumstances may represent an acute medical emergency, prompt and correct diagnosis may have fateful therapeutic consequences.

This in general is true in pediatrics, as most other causes of marked or profound hyponatremia in this age group are associated with dehydration: again, requiring administration of fluid and electrolytes rather than fluid restriction. The pediatric literature is singularly deficient in reporting cases of SIADH, although a consideration of the subject leads us to the conclusion that further search in this age group may reveal many unrecognized cases, with important and frequently emergent therapeutic implications, especially as in this age group (pneumonia, meningitis, etc.) prompt and proper treatment of the hyponatremia may afford the margin of safety until effective antibacterial and other therapy can take effect.

4) Studies of vitamin E in man

Studies of vitamin E deficiency in man and on the effects in humans of megavitamin E supplementation have been completed and are giving rise to numerous publications.

These investigations have been very productive and it has been shown conclusively that vitamin E deficiency in man, using patients with CF as models (as they have profound and long-standing deficiency of this supplement) produces in humans many of the features seen in experimental animals, although usually at a sub-clinical level. Among others, erythrocyte hemolysis is increased, erythrocyte survival is shortened and there are indications of muscle involvement, as shown by creatinuria and, at times, by increases in plasma creatinine-kinase and aldolase. Conversely, on electron microscopy, there are no morphologic changes visible in subcellular organelles in intestinal biopsies. Most of the pathologic findings are corrected, in vitro and in vivo, by administration of vitamin E. On the other hand, normal controls had normal levels of tocopherol in plasma, regardless of vitamin E supplementation. It was concluded that patients with CF and pancreatic deficiency, and other malabsorptive causes of vitamin E deficiency, as well as populations in areas of the world exposed to famine conditions (e.g., Bangladesh, Jordan, Ethiopia, etc.) and receiving inadequate diets, should be supplemented with vitamin E.

In contrast, a study of normal adults voluntarily taking large amounts of this vitamin did not show any benefits from the supplementation, despite significantly higher levels of plasma α-tocopherol. No toxicity effects were noted, probably because the α-tocopherol levels correlate with plasma triglyceride and cholesterol concentrations, and therefore do not increase beyond a certain level, regardless of the amount of vitamin E ingested. There were no specific beneficial effects which were noted consistently. It was concluded that vitamin E supplementation is not necessary or advisable in normal individuals in this and other advanced countries in which the diet is adequate.

Significance to Biomedical Research: Cystic fibrosis in adults needs further definition, as many of the complications seen in the older age group are different and are treated differently from those in children. In particular, pregnancy in CF is hazardous, and necessitates treatment with antibiotics. Much information is being gathered on this point, which has posed a serious problem for physicians. Knowledge of other complications of CF, which have not been previously described, may prove useful for physicians as well--especially those like SIADH, which can easily be confused with another syndrome which requires completely different treatment. The almost universal deficiency of vitamin E in CF and its clinical consequences is of significance in furthering our knowledge of vitamin E function in normal humans, and the utility of the currently popular practice of megavitamin E supplementation in normal humans.

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1. Authors: Gibson, L.E., di Sant'Agnese, P.A. and Shwachman, H.: Procedure for the Quantitative Iontophoretic Sweat Test for Cystic Fibrosis. Atlanta, Ga., Cystic Fibrosis Foundation, 1975, pp 8.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 49003-01-PMB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cyclic Nucleotides and Fucose in Cystic Fibrosis

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

PI:	P.A. di Sant'Agnese	Chief	Pediatric Metabolism Branch	PMB:NIAMDD
OTHER:	P.B. Davis	Clinical Assoc.	Pediatric Metabolism Branch	PMB:NIAMDD

COOPERATING UNITS (if any)

LOM:NIDR R.F. Wolf Dental Director
APB:NIMH J.F. Tallman Acting Chief, Section on Biochemistry

LAB/BRANCH

Pediatric Metabolism Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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

Cystic fibrosis (CF) is a disorder primarily involving the exocrine glands, and we have begun to study cyclic nucleotides, which may be essential determinants of secretion, in CF. Present topics are: 1) the concentration and distribution of cyclic AMP and cyclic GMP in normal and CF body fluids, 2) the response of cyclic AMP to β receptor stimulation in normal and CF lymphocytes and fibroblasts, and 3) the response of cyclic GMP to stimulation of the cholinergic receptor in several systems.

Objectives: The role of the cyclic nucleotides in exocrine secretory processes is still being determined, but it is suspected that they mediate the influence of the autonomic nervous system on the glands and may be essential determinants of secretion. Since cystic fibrosis is a disorder involving predominantly the exocrine glands, we have begun to study the physiology and distribution of cyclic nucleotides in CF. Fucose is a major membrane sugar and we have studied fucose incorporation into fibroblasts.

1. Cyclic AMP and cyclic GMP in submaxillary saliva and urine

Four CF patients and three normal controls of comparable age underwent collection of submaxillary saliva in the fasting state with the aid of a Schneyer segregator. Submaxillary saliva was selected because this predominantly mucous secretion has been shown to be abnormal in CF. Preliminary results indicate that there is little difference in concentration of cyclic AMP (cAMP) and cyclic GMP (cGMP) between normal and CF submaxillary saliva. See chart below:

	Saliva	
	cAMP (pmole/ml)	cGMP (pmole/ml)
Normal N=5	16.2 ± 3.0	1.73 ± .35
CF N=6	19.8 ± 7.0	1.50 ± .24

However, because of the large variation in CF cAMP samples, more patients may be studied, and parotid saliva (predominantly serous secretion) may be collected by the Lashley cup technique and assayed.

Murad et al. have reported abnormalities in cAMP and cGMP excretion in CF, compared to age-matched controls (J Endocr & Metab 10:552, 1975). We have determined cAMP and cGMP in 24-hour urine from adult male CF patients and appropriate controls and have found that older CF patients have slightly increased urinary cAMP, but markedly elevated urinary cGMP. See chart below:

	Urine	
	cAMP (nmole/g creat)	cGMP (nmole/g creat)
Normal N=5	2.23 ± 0.4	0.16 ± 0.01
CF N=6	2.88 ± 0.3	0.28 ± 0.09

Moreover, we are investigating the relation of urinary cAMP and cGMP to the degree of illness as gauged by the NIH Clinical Score. This study is continuing,as more data is required for statistically valid conclusions, and we plan to extend the study to heterozygotes, to determine whether these changes are gene-related.

2. Isoproterenol stimulation of cyclic AMP in lymphocytes and fibroblasts

Canadian workers have shown that CF fibroblasts respond with 3-15 times more cAMP to isoproterenol stimulation than do normal cells. We are currently attempting to study the β receptor and its cAMP response in fresh leukocytes and cultured fibroblasts. The leukocyte assay must first be standardized with respect to the state of the subject from whom the blood is obtained, and we are currently exploring the relation of feeding, exercise and the menstrual cycle to these assays.

3. Fucose in CF

Chou and Nadler reported (Ped Res 9:312, 1975) that normal but not CF serum stimulated fucose incorporation into both normal and CF fibroblasts. We attempted to reproduce these experiments, but found that CF and normal serum both stimulated fucose incorporation by fibroblasts to the same extent, under a wide variety of conditions of handling of serum, fibroblast growth, harvesting and processing of samples. No differences were found in fucose incorporation by CF fibroblasts compared to normals when results were expressed per 10^6 cells; however, since CF fibroblasts contain only 2/3 the protein of normal cells, incorporation of ^{3}H-L-fucose per mgm fibroblast protein was 1.5 times as great in CF cells as normals. Thus, we are unable to confirm the observations of Chou and Nadler with respect to ^{3}H-L-fucose incorporation by fibroblasts.

Significance to Biomedical Research: The process of secretion is only now being elucidated and since cystic fibrosis is a disorder predominantly of secretory glands, it is important to study the various parts of this process to define any possible abnormality.

Publications:

Farrell, P.M., Pallavicini, J.C. and Ulane, M.M.: Growth Characteristics and Protein Content of Tissue-Cultured Fibroblasts from Cystic Fibrosis Patients. Proc Soc exper Biol 149:340-343, 1975.

ANNUAL REPORT SUMMARY

CLINICAL HEMATOLOGY BRANCH

I. Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

1. Effects of Proteolytic Inhibitors on Blood Coagulation

In our previous studies of the effects of proteolytic inhibitors on blood coagulation, we found that the soybean trypsin inhibitor is a potent anticoagulant that prevents prothrombin from being converted to thrombin in a reaction that occurs only after coagulation has been initiated. In an uninhibited, purified activation system containing isolated Factor X, Factor V, phospholipid and calcium, prothrombin forms two major derivatives prior to formation of thrombin. These derivatives, which represent fragments of prothrombin cleaved by activators, are identified primarily by acrylamide gel electrophoresis. When soybean trypsin inhibitor is present in the activation mixture prothrombin derivative formation is prevented at the earliest stages of prothrombin conversion. In our work this year we have found that soybean trypsin inhibitor does not combine with prothrombin or any of its derivatives, combines weakly with Factor X, and not with Factor V or phospholipid. These conclusions were based on solid phase chromatography experiments in which each of the purified clotting factors was linked to Sepharose and radioactively labelled soybean trypsin inhibitor was passed over the columns. In addition, kinetic analyses of the reactions inhibited by soybean inhibitor were compared with the rates and final yields of thrombin obtained when each of the purified clotting factors was progressively limited. These analyses indicated that Factor X was not the limiting factor in reactions inhibited by soybean inhibitor. Instead the soybean trypsin inhibitor appeared to decrease the Factor V activity despite inability to demonstrate this by solid phase chromatography. Since esterolytic and proteolytic activities have been identified as characteristics of Factor X reactions but not of Factor V, the most likely mechanism of trypsin inhibitor activity is that it prevents Factor V from exerting its co-factor activity in association with Factor X. These findings have several implications in the field of coagulation. As yet no specific function has been ascribed to the Factor V molecule; and the demonstration that potent anticoagulant activity is in association with inhibition of this factor suggests new approaches to developing anticoagulant therapy. The type of anticoagulant activity effected by proteolytic inhibitors is especially suited for clinical anti-coagulation because of its marked rate dependence. That is, proteolytic inhibitors tend to effectively block slow rates of prothrombin conversion that are most likely associated with intravascular thrombosis formation, but are relatively less effective in blocking rapid rates of prothrombin conversion that are stimulated by tissue factors, for example, the type of clotting that would occur when blood is extravasated following trauma or surgery.

2. Molecular Structure of Factor VIII

Using gel filtration on Sepharose 4b following limited digestion of plasma with chymotrypsin we found that Factor VIII could be separated from

plasma in a single column passage by virtue of its extremely high molecular weight. Factor VIII appeared as an isolated peak in the void volume while almost all other proteins eluted much later. We further found, along with other investigators, that the approximately two million molecular weight Factor VIII material could be separated into at least two components, one remaining essentially the original size and the other, which contained the procoagulant activity, separating under the influence of high ionic strength or relatively high concentration of ionic calcium as a retarded peak appearing to be in the 100-200,000 molecular weight range. Because of the slow filtration rates on Sepharose 4b most of the procoagulant activity was lost even when potent highly purified starting material was used. Because of this, evaluation of the separation of Factor VIII components was at best tenuous and qualitative as well as quantitative comparisons between normal plasma and plasma from patients with von Willebrand's disease and classic hemophilia were meaningless. These comparisons are of importance because they hold the key to the molecular abnormalities involved in the two diseases.

During the past year we developed a rapid and simple method of separating Factor VIII from plasma or partially purified concentrates that does not require the chymotrypsin digestion step. By using inorganic porous filters it was possible to accelerate the separation of high molecular weight Factor VIII by approximately two orders of magnitude compared to its separation on Sepharose. Moreover because the filters do not compact, the inevitable debris present in plasma does not cause clogging; and fibrinogen which tends to aggregate and precipitate spontaneously at room temperature or in the cold does not interfere with separation as it does when Sepharose is used. In order to utilize inorganic porous filters it was found necessary to coat them with appropriate organic compounds to prevent nonspecific adsorption of Factor VIII. Once the method was established purified Factor VIII could be separated from plasma in one passage with a procoagulant yield approaching 100% compared to 1-5% yield with other methods. With the new technique it has been possible to determine within a precise range the ionic strength and cation requirement necessary to dissociate Factor VIII into large and small molecular moieties. Because separation is fast and yields of procoagulant activity are high, for the first time it has been possible to determine antigenic differences in the two species of Factor VIII utilizing antibodies raised in rabbits against the different Factor VIII components as well as naturally occurring antibodies in treated hemophiliacs and patients with acquired inhibitors of the idiopathic variety. From these studies it is apparent that the acquired inhibitors in patients react only with the procoagulant (small molecular weight component) of Factor VIII whereas animal anti-Factor VIII reacts with the high molecular weight component. Hemophiliacs apparently lack the coagulant portion of the molecule in that human antibodies are not blocked by hemophilic plasma or Factor VIII component separated from hemophilic plasma whereas animal antibodies react with hemophilic plasma or the separated components just as does normal plasma. The small coagulant portion of the Factor VIII molecule after separation from the large component at high ionic strength was found to recombine by simply lowering the ionic strength to physiologic levels. Although the amino acid analysis of the high molecular weight Factor VIII including the procoagulant moiety has been reported by us and others, there has been as yet no analysis of the isolated

small component that appears to be the most important part of the anti-hemophilic factor in coagulation. Our current efforts are directed at accumulating this small component which is at such low concentration that it cannot be detected by protein analytic techniques at levels far above that present in normal plasma. The method of separation that we have developed is the only one currently available that potentially will permit separation of sufficient small molecular weight material for analysis.

"Platelet Survival and Morphology in Homocystinuria Due to Cystathionine Synthase Deficiency"

Patients with an inherited deficiency of cystathionine synthase have elevated levels of homocystine in the plasma, increased urinary homocystine; and among other clinical manifestations, susceptibility to arterial and venous thromboembolism. Because thromboembolism is a major cause of mortality and morbidity in this disease, patients have been prophilactically treated with antithrombotic agents directed at inhibiting platelet function although there is no clear evidence that a platelet abnormality exists. Two recent reports in the literature suggest in one, that platelet survival in homocystinuric patients is markedly decreased; and in the other, that platelets of patients with homocystinuria have increased vacuolization as seen by electron microscopy. Ten years ago we studied one of the first reported cases of cystathionine synthase deficiency and found that platelet survival in that individual, despite the presence of thromboembolic phenomena, was normal. Because of the discrepancy between this finding and those recently reported we repeated the studies of platelet survival in five additional patients and also examined their platelets by electron microscopy. In all of the patients that we studied platelet survival was entirely normal and platelets from five homocystinurics and three obligate heterozygotes could not be distinguished from seven normal control subjects by electron microscopy. Specifically, no increased vacuolization was observed. The mechanism of thrombosis in homocystinuria is therefore still open to question. Because there has been a proliferation of reports suggesting that minor abnormalities of homocystine metabolism may be the basis of arteriosclerosis and thromboembolic phenomenon in otherwise normal individuals, we feel that our finding that homocystinuric patients have normal platelet function and morphology is important to help clarify the present confused state of information on the subject.

II. Study of the Immunology of Blood Cell Deficiencies

1. The Effect of Platelet Aggregating Agents on Cyclic Nucleotide Metabolism

In studies over the past few years we have found that agents which cause platelet aggregation and release of platelet granules markedly decrease adenylate cyclase activity but do not significantly change cyclic-AMP levels of platelets. Because of its importance in a variety of clinical conditions, we have studied the effects of antiplatelet antibodies as aggregating agents, evaluating the effects of these antibodies on guanylate cyclase and cyclic-GMP

and comparing the antibodies with other aggregating agents such as thrombin, ADP, arachidonic acid, and epinephrin. It was found that the activity of platelet guanylate cyclase during aggregation depends on the nature and mode of action of the inducing agents. Aggregation induced by thrombin and ADP increased guanylate cyclase activity whereas arachidonic acid and epinephrin caused a marked decrease in guanylate cyclase activity. The effects of antibodies were more like those of thrombin and ADP, but it was not possible to obtain consistent results with antibodies. Preliminary investigations indicate that a variety of unsaturated lipids cause stimulation of guanylate cyclase, the degree of stimulation roughly paralleling the degree of lipid unsaturation. It is possible that unsaturated fatty acids in platelet membranes are made more available by the action of thrombin, ADP or antibodies when they act to cause platelet aggregation. Some of these unsaturated fatty acids may be involved in the initial phases of prostaglandin biosynthesis which is accelerated during platelet aggregation in response to ADP, thrombin, and antibodies. So far it has been difficult to reconcile these observations with the marked decrease in guanylate cyclase that occurs with arachidonic and epinephrin induced platelet aggregation; but further studies of cell-free systems and further knowledge of the effects of intermediate endoperoxides in arachidonic acid metabolic pathway may be helpful in resolving this discrepancy.

2. Heparin-induced thrombocytopenia

Development of thrombocytopenia is a relatively common occurrence during heparin therapy. Among the various possible causes of thrombocytopenia are the primary disorder such as intravascular coagulation for which heparin is given, the direct effect of heparin through a reaction with proteins such as fibrinogen on the platelet surface, and immune reactions involving possible anti-heparin antibodies. During the past year we have prospectively studied 52 patients undergoing heparin therapy at Johns Hopkins Hospital in a collaborative effort. Pre-treatment, treatment, and post-treatment samples were obtained on 35 individuals, 16 of whom developed varying degrees of thrombocytopenia while on therapy. Using a sensitive serotonin release test developed in this laboratory for detecting low levels of anti-platelet antibody we were unable to demonstrate in any instance anti-heparin antibody in those individuals who developed thrombocytopenia. In addition, classical methods of measuring drug antibodies including complement fixation and agglutination were negative. Of special interest was the finding that those individuals who tended to develop thrombocytopenia had in their plasma a component that augmented serotonin release from platelets prior to initiation of heparin therapy. It appears that under certain circumstances abnormal globulins may develop during the course of disease that, in association with heparin, may lead to coating of platelets in a non-specific manner and possibly promote thrombocytopenia. Although the literature contains a number of articles suggesting that specific antibodies against heparin are involved in the thrombocytopenia that develops, we have conclusively shown that this is not the case; and that other factors, as yet unknown, must be the basis for the disorder.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 51,000-19 CHB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Study of Immunology of Blood Cell Deficiencies

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

PI: N.R. Shulman Chief, Clin. Hematology Br. CHB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	3/4	1 1/4

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

Autoimmunity, drug antibodies, platelet antigens, isoimmunization, transplantation antigens, hepatitis, hemagglutination tests, thrombocytopenia, blood coagulation inhibitors

877

PHS-6040
(12-75)

Project No. Z01 AM 51,000-19 CHB

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. Immunologic techniques have been used to develop a number of clinically practical diagnostic tests for certain infectious diseases and drug addiction.

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, immunoelectrophoresis, methods of provoking antibody responses in man and animals, and isotopic and flourescent labeling applied to antigens and antibodies. Tissue culture techniques, red cell agglutination and techniques and lymphocyte transformation tests, including radioautography. Methods of hemagglutination involving attachment of various antigens to erythrocytes by chemical coupling.

Publications:

1. Sarna, G., Tomasulo, P., Lotz, M.J., Bubinak, J.F. and Shulman, N.R.: Multiple neoplasms in two siblings with a variant form of Fanconi's anemia. Cancer. 36: 1029-1033, 1975.

2. Shulman, N.R.: Analyses of drug antibodies with autoimmune implications. In Vyas, G.N., Stites, D.P. and Brecher, G. (Eds.): Laboratory Diagnosis of Immunologic Disorders. New York, Grune & Stratton, Inc., 1975, pp. 31-46.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 51,001-19 CHB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

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

PI: N.R. Shulman Chief, Clin. Hematology Br. CHB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	3/4	1 1/4

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

Hemophilia, coagulation mechanism, clotting factor assays, clotting factor purification, platelet physiology, platelet metabolism

PHS-6040
(12-75)

Project No. Z01 AM 51,001-19 CHB

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 radioactivity labeled substrates.

Publications:

1. Gordon, N.R. and Shulman, N.R.: Discussion paper: The effect of clotting on structure and function of human Factor VIII. Ann. N.Y. Acad. Sci. 240: 79-83, 1975.

2. Kasper, C.K., Aledort, L.M., Counts, R.B., Edson, J.R., Frantantoni, J., Green, D., Hampton, J.W., Hilgartner, M.W., Lazerson, J. Levine, P.H., McMillan, C.W., Pool, J.G., Shapiro, S.S., Shulman, N.R. and van Eys, J.: A more uniform measurement of Factor VIII inhibitors. Thrombos. Diathes. haemorrh. (Stuttg.). (Letter to the Editor) 34: 869-872, 1975.

3. Uhlemann, E.R., TenPas, J.H., Lucky, A.W., Schulman, J.D., Mudd, S.H. and Shulman, N.R.: Platelet survival and morphology in homocystinuria due to cystathionine synthase deficiency. N. Engl. J. Med., in press.

OFFICE OF THE
ASSOCIATE DIRECTOR FOR
EXTRAMURAL PROGRAM ACTIVITIES

During FY 1976, further advances were made toward implementation of a Program-Cluster approach and toward the adaptation of Extramural Program Activities to the many requirements and changes imposed by the transition. Most noteworthy have been the continued strengthening of the program/cluster concept within the Offices of the Associate Directors for the Digestive Diseases and Nutrition Program and the Kidney, Urologic, and Blood Diseases Program, and the promise of appointment of Associate Directors for the Arthritis, Bone, and Skin Diseases Program and the Diabetes, Endocrine, and Metabolic Diseases Program in July 1976. The Office of the Associate Director for Extramural Program Activities has been deeply involved in the preparations for the arrival of these latter individuals and in the planning for an orderly transition to full expression of the Program-Cluster concept.

The cluster concept has also been reflected in the meetings of the National Arthritis, Metabolism, and Digestive Diseases Advisory Council. Subcouncils have been constituted for each of the cluster and during FY 1976 met separately on the first day of each of the scheduled National Advisory Council meetings. They have provided support to cluster program staff in planning and giving special consideration to particular program emphasis and initiatives.

In addition, in FY 1976, efforts were made to improve the Council meetings. Of particular note has been the increased utilization of staff recommendation forms for those applications requiring Council's special attention. These documents are provided to members of the Council, before each meeting, and state the reason why Council's special consideration is warranted together with the various options available to Council for action. The net result has been a decrease in the amount of time Council spends on such matters.

Over the year, the objectives of the Office of the Associate Director for Extramural Program Activities have been steadily evolving and expanding in the directions of (1) planning and defining for improved utilization of research-support mechanisms; (2) improving the capability for program analysis, evaluation, planning, and development, and (3) strengthening the administration of all extramural operations and, (4) defining mechanisms and procedures to insure effective communication between Extramural Program Activities staff and the staffs of the Program Clusters.

With respect to the first objective, (a) a committee has recently completed its work on the development of guidelines, announcements, and review procedures for the large-grants (i.e., program project, core center, and center mechanisms grants); (b) a second committee is revising the guidelines for consideration for use of the Clinical Investigator and Academic Investigator Awards by all the program areas, and (c) the staff in the immediate Office of the Associate Director have been actively involved in the development of sorely needed procedures and guidelines for planning, implementation, and review of contract initiatives in the extramural programs.

In the area of improving analysis, evaluation and planning for program development, this Office served as a focal point for the development of a two-day work session for staff and representative Council members. In addition, several extramural staff meetings were entirely devoted to consideration of program analysis, evaluation, and development as carried out by other Institutes. It is intended that these initiatives will result in the development of specific plans for analysis, evaluation and planning by each cluster.

Activities relating to improved administration, cooperation and communication in extramural operations, have focused around the development of EP-Operating Procedures. During FY 1976, issuances which spelled out operating responsibilities and procedures with respect to: (a) awards for foreign institutions; (b) rebudgeting of grant funds; (c) inactivation of applications (d) review of conference grant applications; and (e) release of review information to applicants were developed.

It should be noted that staff of the various Program Clusters participated with EPA staff in all of these areas. In addition, a number of these activities were greatly enhanced by the participation of a Grants Associate or of a Management Intern.

Administrative Office

The Administrative Office staff was heavily involved in meeting the increased demands placed on it by both the National Diabetes Commission and the National Arthritis Commission, particularly in the area of travel. Similiar demands were also placed by the ad hoc training review committees for most of FY 1976. The addition of a temporary employee during FY 1976 in the Administrative Office helped markedly in the operation of the Office. The continuation of this temporary position, and the addition of one more employee during the next year will be a necessity with the expected increase in requests for services including, procurement activities, space management, timekeeper functions and personnel actions associated with the further establishment of the Offices of the Associate Directors.

Program Evaluation Branch

The Activities of the Program Evaluation Branch during FY 1976 can be considered in terms of those that relate directly to the analyses and evaluation functions, and those which relate to the processing and retrieval of data on grant activities in the extramural programs.

Program Analysis and Evaluation

An assessment of current efforts, resources allocated, and progress towards the objectives and mission of NIAMDD is crucial for effective administration and development of extramural programs. The following is a summary of activities in this area:

(1) A comprehensive study to obtain an overall perspective of the extramural programs was initiated. This analysis would provide the necessary base for consideration of alternatives in the development of an effective policy for future resource allocation.

The objective is to compose a picture of the patterns of funding for research and for the development of manpower by NIAMDD as occured in the recent past. The emphasis is on the general trends--a current dynamic process; which is in contrast to the usual emphasis on listing--a static view of past events. Time series data cover the period FY 1969-1974. Some data on FY 1975 are also included.

(2) A corresponding study of individual programs is also in progress; analyses of Arthritis, Dermatology, Diabetes, Endocrinology, Nutrition, and Gastroenterology have been completed. Others are at various stages developing methods for such a study was also a prime objective.

(3) A "Workshop on Analyses, Evaluation, and Planning" was held in April 1976. The emphasis was on exploration of various approaches towards evaluation of the scientific content of extramural programs and their responsiveness in meeting their objectives.

(4) A preliminary exploration of various information, collection, and retrieval systems pertaining to various mechanisms of support and the grantees has been initiated. Activites under 1, 2, 3, and 4 above are expected to provide the necessary framework for the needs of NIAMDD Extramural Programs in their efforts to modify existing operational activities in this area.

(5) For the evaluation of Extramural Programs a general framework based on the mission of the NIAMDD, and the objectives of various clusters and programs, e.g., research and development into the etiology, pathology, diagnosis and treatment, prevention and control of certain diseases, and basic research in related areas, and the training and development of scientists is being developed.

(6) For the deliberations and projections of the Research Work Group and the Epidemiology Work Group of the National Commission on Arthritis, a study was undertaken to assess the resources allocated for the support of research and training under the Arthritis Orthopedics Programs of NIAMDD.

Another study was undertaken to look closely at the distribution of financial support by other institutes of NIH and other Federal agencies for research on arthritis related clinical disorders according to the classification of rheumatic diseases by the Arthritis Foundation.

(7) The design and capabilities of about eight large computer-based systems of importance to the Data Work Group of the National Commission on Arthritis were studied and summarized. This background information was considered necessary in formulating recommendations for the establishment of a national data bank pertaining to arthritis as required by the National Arthritis Act

Data Activities

The Program Evaluation Branch has assigned and processed 2,141 competing applications and scientifically coded the awarded research and career applications, including 1,582 noncompeting applications. Due to the release of impounded funds late in 1974, most of the noncompeting applications had to be processed in the last quarter of 1975 putting a strain on the staff.

A remote computer terminal (2741) was added during the year to facilitate the operations. It is being used to set up tables, update files, and to key-in recurring reports.

An NIH ADP/EP Coordination Committee was established. It includes a voting member from each of the NIH BID's and other Agencies that utilize the IMPAC system. The Assistant Branch Chief has represented NIAMDD-EP at the monthly meetings. They are also attended by AM's Computer Specialists. This committee has accomplished better communication between the institutes and especially between the institutes and DRG. It has also recommended some changes and additions to the IMPAC system.

Reports were prepared for the use of staff and our National Advisory Council members for each of the council meetings including a new report showing the interdigitation of council applications with previous competing but unfunded ones by Program Area, in priority order. Monthly recurring reports covering encumbered grants, active grants, noncompeting applications and competing applications were routinely distributed to staff.

Special requests for reports were prepared for staff, other institutes, the Arthritis Commission, other agencies and congressional inquiries. Examples of these follow:

- Report for Rand Corporation covering 1973 Program Projects and scientific classification of all NIAMDD research grants, needed for their NIH contract NO1 OD 52127

- History of the Institute's support in the various arthritis and orthopedic diseases and basic research categories covering 1970 through 1975 for the Arthritis Commission

- Report for the Orthopedics Program Director to present to the American Academy of Orthopedic Surgeons, showing the spread of Initial Review Group (IRG) recommendations, and dollars awarded for fiscal year 1975

- Completed the annual report of population research supported by NIAMDD for NICHD's Inventory of Population Research

- Reported the 1975 obligations for subprogram requested by the Budget Office, e.g., on Psoriasis, Cystic Fibrosis, Sickle Cell Anemia, etc.

- Special report on Diabetes research being supported in all of the extramural program in FY 1975

- History of the Institute's funding of research in Thyroid disease.

The Program Evaluation Branch coordinated the institute's response for the NIH Inventory of Clinical Trials and the assistant Branch Chief was on the NIH Clinical Trials Technical Committee. The Program Evaluation Branch also coordinated the gathering of AM data for the Interdepartmental Task Force on Research in Aging.

Some staff of the Branch attended STEP modules or classes: Program Planning, Analysis and Evaluation, Introduction to NIH Extramural Programs, COBOL Workshop, Grants and Contracts, and Interagency Agreements.

The staff of the Program Evaluation Branch has done yeoman work throughout the year, often working long hours of overtime to keep from falling too far behind or to prepare special reports. In spite of serious understaffing, the Branch has managed to complete the essential tasks, although not always in a timely manner. It is feared that if the workload stays at the present level, some of the important jobs will fall further behind, for example scientific coding and preparation of council reports and others will have to be dropped.

Grants Management Branch

Fiscal year 1976 produced no startingly new developments in the area of grants management; but it did allow certain refinements of old developments. In particularly, the issuance of EP-Operating Procedures has resulted in the delineation of responsibilities for GMB in such areas as rebudgeting of grant funds. The new procedures allows for much closer GMB involvement in the rebudgeting and other processes.

Another refinement concerns the adaptation to the Program Cluster concept. In FY 1975, individual Grants Management Specialists were made responsible for the work relating to each program cluster. Initially this was accomplished for the processing of competing applications. During this past year, this assignment of responsibilities was expanded to noncompeting applications. Although the new procedure is still "shaking down", there is every indication that the desired result of closer cooperation between GMB and the clusters is being achieved.

Once again, the annual appropriation struggle delayed competitive funding decisions until the last quarter of the fiscal year. Combined with the last several years experience, this has had the effect of making a disproportionately large number of our awards carry April, May, and June start dates. With such a large percentage of our dollars remaining unencumbered for most of the fiscal year, the process of closing out the books on the fiscal year was made that much more difficult. The fact that the Federal fiscal year is changing from July 1, through June 30, to October 1, through September 30, effective this summer will hopefully help to relieve this problem for the future.

Planning for the Transition Quarter (TQ) between fiscal 1976 ending June 30, 1976 and the beginning of fiscal 1977 on October 1, 1976 has been an extra task this year. Estimates of fund requirements for the TQ had to be made early in fiscal 1976. Since that time we have been involved with planning the use of TQ funds to enable the transition to proceed smoothly.

The personnel situation within the branch has worsened during the year. Although the number of people has remained constant, the duties and responsibilities of the branch have expanded. This is evidenced by the continuing increase in Grants Management responsibilities and results in the need for additional highly qualified Grants Management Specialists. It is also true in the need for additional secretarial help. A measure of the expertise and commitment of the personnel of the branch is shown by the recent awarding of a Group Special Achievement Award.

Grants Operations

When the standards for disposal of NIH grants case files were revised in September 1975 terminated grant files for FY 1969 and earlier were destroyed. In addition, the files for applications from the same period have also been destroyed. The result of this formidable task has been a freeing of shelf space for about 10,000 grant files.

OFFICE OF THE ASSOCIATE DIRECTOR
For
DIGESTIVE DISEASES AND NUTRITION

ASSOCIATE DIRECTOR'S REPORT

Extensive analyses of opportunities and needs in research in digestive diseases was conducted by 300 scientists and has recently been published in Gastroenterology. Comments from the research community on these analyses are now being reviewed by a new committee that is also reassessing the report as a whole. This group is looking for areas that are important and that offer fruitful opportunities for research with the technology that is already available. They will propose methods of stimulating the needed research, for example, by identifying areas in which workshops could be helpful.

The committee on risks in human experimentation concluded that the data available in published reports was inadequate. Accordingly, the committee is attempting to obtain prospective data. It is beginning with studies on the risk of colonoscopy and of biopsy of the colon. It plans to ask investigators who employ these techniques to set up a system for identifying and tabulating the morbidity and mortality of the procedures. The NCI is helping with this effort.

While the digestive tract is the most common site of cancer, there has been relatively limited involvement of Gastroenterologists in therapy, teaching, and research in cancer. A workshop is being planned in cooperation with the NCI to examine the need for training of gastroenterologists in cancer.

Dr. Karl Mason retired from his position as program director for Nutrition. Dr. Gerald Combs has become the new program director. Dr. Combs was Head of the Department of Nutrition at the University of Georgia.

A committee coordinating the nutrition activities in the NIH has been organized under the chairmanship of the Associate Director for DD-N. It has provided a means of exchanging information on workshops, conferences, and other activities among the institutes. The committee is also working on a common system of reporting grants and contracts that will enable investigators to easily identify relevant research regardless of the institute supporting it.

A workshop on nutritional fiber is being planned through the Nutrition Coordinating Committee. A number of institutes are concerned with the health implications of nutritional fiber: NHLI, NCI, National Institute of Environmental Health Sciences, and NIAMDD.

DIGESTIVE DISEASES PROGRAM AREA

During Fiscal Year 1975, the plan to reorganize operationally the Digestive Diseases Program Area was implemented. All elements of the extramural program pertaining to liver disease, biliary tract disease, and pancreatic diseases were relegated to the newly created program area titled Liver Diseases Program Area under the segis of Dr. Sarah Kalser as Program Director. Consequently, the Digestive Diseases Program Area is now responsible for the extramural support of grant awards and contracts pertaining to certain organ systems of the gastrointestinal tract. These include the esophagus, stomach and duodenum, and small and large intestines. Also included in this program area are studies of the cause, prevention, diagnosis, and treatment of diseases of these organ systems such as achalasia, esohageal reflux, ulcer disease, colonic and inflammatory bowel diseases, and the psycho-social aspects of digestive diseases. Finally, support is provided for research, development, and evaluation of techniques, methods or approaches in the use of endoscopic and other instrumentation for the diagnosis, treatment, and investigation of relevant digestive diseases.

A. Research Grants

During FY 1975, as previously, much of the activity supported by research grants was concerned with the basic aspects of the physiology and biochemistry of disease entities. Of these, many have clinical implications although the studies were performed in animal models. Several projects were clinical investigations whose results are directly applicable to human disease. Of the considerable amount of data and information reported by the grantees during the past year, only a few examples will be cited as representative of the progress that has been made in the Digestive Disesses Program Area.

1. Parenteral Feeding and Gastrointestinal Function

Total parenteral nutrition via intravenous alimentation has been employed increasingly by hospitals in recent years to feed seriously ill patients. Yet little is known about the effects of the regularly occurring presence of food in the gut on gastrointestinal structure and function.

Two separate teams of investigators have reported similar findings. Intravenous liquid feeding is associated with decreases in gastrin levels, disaccharidase activity, and the tissue weights of the oxyntic gland area of the stomach, pancreas, and small intestine. These changes can be largely prevented if the deficient hormone, gastrin or even pentagastrin, is supplied exogenously. Similiar changes did not occur when an isocaloric oral diet was fed. Thus, oral ingestion of food is apparently necessary to maintain the structural integrity of certain tissues of the gastrointestinal tract, and maintenance of normal tissue

stores of gastrin may depend on stimuli provide by oral ingestion of food. Moreover, the trophic effect of gastrin may be necessary to maintain the structural and functional integrity of the gut. Although these changes were noted in animals, similar effects may occur in human patients fed parenterally.

2. Gastric Ulcer Disease

Cimetidine is an H_2 - receptor antagonist and a number of a new class of antihistamines. Reports are now appearing to show that this new drug is a potent inhibitor of gastric acid secretion with no demonstrable side effects. Thus far, the results have been dramatic and will inhibit basal and meal stimulated acid secretion in the stomach in patients with duodenal ulcers and also with the Zollinger-Ellison syndrome.

With the use of cimetidine, active ulcers heal rapidly. Moreover, this drug appears to increase the gastric mucosal potential difference which is related to the gastric mucosal barrier. Cimetidine, therefore, may have a protective effect on the gastric mucosal barrier in addition to its suppression of acid secretion. If these results are validated in other studies, cimetidine could also be of significant importance in protecting patients being treated with aspirin which is alleged to break the mucosal barrier. Chronic aspirin users and rheumatoid arthritics would benefit from its use and obviate the formation of gastric ulcer and acute erosive gastritis with subsequent bleeding.

3. Inflammatory Bowel Disease

a. Naturally occurring lymphocytotoxic antibodies have been found in the serum of patients with Crohn's disease or chronic ulcerative colitis. The prevalence of lymphocytotoxic antibodies in both forms of inflammatory bowel disease was found to be about 40 per cent. The cause of chronic inflammatory bowel disease remains unknown; however, recent studies have implicated a transmissible agent as the causative factor. To further substantiate this hypothesis, a study was recently completed to determine the frequency of lymphocytotoxic antibodies found in family members of individuals inflammatory bowel disease. These family members were further divided into groups described as household contacts and non-household contacts. Data obtained from family members were compared with a matched normal population. Results of this study clearly demonstrates an increased prevalence (30 per cent) of naturally occurring lymphocytotoxic antibody in the family members of probands with inflammatory bowel disease as compared to a matched normal population (4 percent). In addition, the prevalence of the antibody in household contacts of the propands was greater (40 per cent) than the nonhousehold contacts (19 per cent). These findings again suggest that an environmental or common agent may be responsible for either Crohn's disease or chronic ulcerative colitis.

b. The treatment of inflammatory bowel disease which includes Crohn's disease and ulcerative colitis has essentially been empirical. The more commonly used drugs are prednisone and/or sulfasalazine with the occasional use of azathioprine. The response of the patient has been largely subjective. Decreased incidence of pain, decreased frequency of diarrhea, weight gain, general well being are the usual criteria used to measure the success of therapy. Clinical improvement may also be assessed by sigmoidoscopy and X'ray examinations.

Recent studies have been completed which may offer a more definitive approach to the assessment of the severity of the disease process in Crohn's disease, ulcerative colitis, and celiac disease. The presence of antibodies to orally administered bovine serum albumin (BSA) was determined in the serum of normal subjects and patients with inflammatory bowel disease and celiac disease. Antibodies to BSA were demonstrated in 28 of 30 patients with ulcerative colities (93%), 30 of 35 with Crohn's disease (86%), 5 with untreated celiac disease and in 12 of 28 normal subjects (43%). In patients with inflammatory bowel disease, antibodies to BSA were present in greater amounts in those with severe and moderate disease than in those with mild disease. Moreover, in those patients with high titers of circulatory antibody, the serum anti -BSA activity was always associated with IgG which suggests that an increased absorption of antigenic material and stimulation of antibody production may occur in association with intestinal mucosal damage.

4. Irritable Bowel Syndrome

Irritable bowel syndrome reportedly accounts for half of the gastrointestinal complaints brought to the attention of physicians and ranks second as a cause of industrial absenteeism due to illness. Despite numerous investigations, no organic cause for this syndrome has been discovered, although the coexistence of "nervous manifestiations" is implied.

To further clarify this issue and obtain additional data to correlate psychiatric illness with the irritable bowel syndrome, a study was carried out through the collaborative efforts of psychiatrists and gastroenterologists. Twenty-nine out-patients with irritable bowel syndrome were given structured psychiatric interviews, as were 33 medical controls who did not have irritable bowel syndrome. Seventy-two percent of the irritable bowel syndrome subjects had psychiatric illness, with hysteria and depression the most prevalent syndromes. Only 18% of controls had psychiatric illness. The primary physican made an accurate psychiatric assessment in only 28% of the subjects.

The results achieved by this study has practical and significant implications for the primary physician. It was not only confirmed that patients with irritable bowel syndrome (IBS) have a high prevelence of psychiatric illness, with psychiatric symptoms, but it also points out the necessity for primary physicians to screen each patient with IBS for psychiatric illness, particulary hysteria and depression. As a consequence, numerous patients would be spared needless medications, hospitalization, and surgery.

B. CONTRACTS

1. National Cooperative Crohn's Disease Study
Contract No. NO1 - AM-2-2210
University of Colorado, Denver, Colorado
Total duration - 5 years Total Cost - $1,456,346

During the past four years the NIAMDD has been sponsoring the National Cooperative Crohn's Disease Study (NCCDS). At 14 study centers NCCDS studied two aspects of the response of Crohn's Disease to azathioprine, prednisone, sulfasalazine, or placebo: control of actively symptomatic disease during 4 months of high-dose treatment (Part I) and prevention of flare-up or recurrence of quiescent or surgically resected disease by low-dose treatment (Part II). An eight-item Crohn's Disease Activity Index (CDAI) was developed as the primary measure of response or recurrence. In part I, 234 actively symptomatic (CDAI 150) patients have been followed at weekly, then bi-weekly intervals for 4 months after random allocation to one of the following daily regimens: prednisone on a sliding dosage scale from 1/4 to 3/4 mg/kg body weight, depending on CDAI; sulfasalazine 15 mg/kg body weight; azathioprine 2.5 mg/kg body weight; placebo. Patients and evaluating physicians remained blind to the nature of the regimen. Groups of patients randomized to each of the 4 regimens are comparable in all aspects. At the present time, nearly 600 patients have been randomized into the study and 400 of these are off-study. During the following year, the computerized data will be analyzed and results will be published thereafter. It is anticipated that one or more of the test drugs will be found to be superior to placebo in control of active symptomatic Crohn's disease.

Plans are being formulated to conduct additional studies of drugs in combination with particular emphasis in determing the "drug sparing effect" of one in relation to another. The same basic protocol and Activity Index will be used.

These studies have been conducted under the direction of Drs. Fred Kern, Jr. and John Singleton of the University of Colorado.

2. Research and Development Studies in Gastrointestinal Endoscopy

a. Contract NO: NO1 - AM-5-2222
Title: Control of GI Tract Bleeding by Laser Photocoagulation
Institution: Beth Israel Medical Center, New York, New York
Duration: One Year
Contract Amount: $99,120

b. Contract NO: NO1-AM-5-2211
Title: Endoscopic Control of Continued Massive Gastric and Duodenal Bleeding in a General Hospital Population
Institution: University of Washington, Seattle, Washington
Duration: Two years
Contract Amount: $ 242,660

c. Contract No: N01-AM-5-2213
Title : 1. Endoscopic Retrograde Cholangio-pancreatography
2. Endoscopic Electrosurgery for Control of Upper G.I. Hemorrhage
Institution: Universtiy of Minnesota, Minneapolis, Minnesota
Duration: Two Years
Contract Amount: $119,609

It is recognized that more effective approaches to the diagnosis and treatment of digestive diseases should lessen the morbidity and economic loss caused by these illnesses. One of the most promising new approaches is endoscopy, a method which provides direct access to the interior of the esophagus, stomach, duodenum, colon, etc, The pancreatic and biliary tracts have recently become accessible via a special duodenoscope.

Currently, three contract studies are being supported to study the control of gastrointestinal bleeding by endoscopic systems involving laser coagulation, electrocoagulation, suture clips, and tissue glues. Also, included in these is a minor project to improve techniques of cannulation of the biliary and pancreatic ducts in conjunction with endoscopic retrograde cholangio-pancreatography,

These studies are being conducted at institutions where the requisite expertise and skills in endoscopy, clinical gastroenterology, and engineering are available. One effort is at the University of Washington Seattle, Washington under the direction of Drs. Fred Silverstein and Cyrus Rubin. A second effort is at the University of Minnesota, Minneapolis, Minnesota under the direction of Dr. Jack Vennes, and the third is at the Beth Israel Medical Center, New York, New York under the direction of Dr. Albert Waitman.

For such studies, it has been stipulated that feasibility, safety, and applicability to diagnosis and treatment must be paramount. All components of instrument systems must be non-toxic and harmless to the human subject. Furthermore, all systems and techniques must be applied to and tested in an appropriate animal model before proceeding to studies with human subjects.

Liver Diseases Program Area
(1975-1976)

During the past year, the Liver Diseases Program Area has been separated from the rest of the Digestive Diseases Program within the Digestive Diseases and Nutrition Program for programmatic and administrative reasona. Research activity in the Liver Diseases Area is concerned with the physiology, biochemistry and morphology of disease entities involving the gallbladder and biliary tract, the liver and the exocrine pancreas. Some of the diseases within this area include gallstones, biliary atresia, cholestasis in adults, acute and chronic liver diseases of both inflammatory and metabolic origin, acute and chronic pancreatitis.

A. Research Grants

Of the many significant advances made in the Liver Diseases Program Area during the past year by Institute-supported grantees, only a few examples will be cited. These deal with the following disorders:

1. Primary Biliary Cirrhosis
2. Hepatic Coma
3. Testosterone Metabolism by the Liver
4. Gallstone Formation in Obesity
5. Diagnosis of Acute Cholecystitis

1. The Effect of D-Penicillamine on Urinary Copper Excertion in Primary Biliary Cirrhosis (PBC)

Primary biliary cirrhosis (PBC) is a fatal disease with a mean survival time estimated as six years. The small bile ducts within the liver are progressively destroyed, perhaps by an immune reaction, causing severe jaundice and intense itching. Cirrhosis develops late in the course of the disease. It may follow a prolonged bout of heptitis. Unfortunately, an effective therapy has not yet been found, although steroids and immunosuppressive agents each have their enthusiasts.

Levels of copper elevated some 30 times over normal have been found in the liver of patients who have PBC, similar to the finding in another disease affecting the liver (and other organ), Wilson's disease. In this later disease, increasing the excretion of copper by giving a drug that tightly binds copper and allows it to be excreted in the urine, has become standard treatment. Because of the similar findings, a pilot study was performed administering D-penicillamine to patients having PBC. These patients showed a sustained increase in their urinary excretion of copper during the 12 months of study. Now these same investigators at the Mayo Clinic propose to systematically study a large group (150) of such patients, one-half of whom will receive placebo and one-half the drug. All of the patients will be asked to adhere to a diet low in copper. The patient will need to be studied for about 5 years and survival will be the ultimate measure of success.

2. Hepatic Coma

Fulminant hepatitis is a relstively rare disease in which hepatitis viruses and certain drugs are presumed causes. Hepatic coma is a frequent terminal episode when the liver has massive areas of cell death (necrosis). The patient may pass from several days of confusion into a stupor and finally, coma. Treatment is aimed at replacing the liver's function until regeneration can occur. Although the cause of the coma is not established, two basic ideas prevail; (1) that the liver is not able to remove toxic substances and these harm brain function or (2) the liver is unable to produce a substance that the brain needs. The first hypothesis is currently thought to be the most likely.

A grantee at Massachusetts General Hospital has been able to prepare dogs in such a way that they can serve as a model for hepatic coma. This grantee first showed that in both man and dogs, there is a ratio of about 3 for branched chain to neutral amino acids in the normal situation. In hepatic coma, this ratio drops to 1.0 showing a decrease in the branched chain amino acids (valine, leucine, and isoleucine). The postulate that these two groups of amino acids (branched chain and neutral) compete for entry into the brain. In hepatic coma, more of the neutral amino acids can gain entry since the plasma level of branched chain acids decreases, and they postulate that this causes deleterious changes in the brain neurotransmitters. They have been successful in correcting the plasma ratio back to normal in dogs by infusing a mixture of branched chain amino acids and this has been associated with improved survival and few neurological symptoms.

They are now attempting to perfect the infusing solution so that it may prove suitable for treatment of patients with hepatic coma.

3. Prolonged Alcohol Consumption Increases Testosterone Metabolism in the Liver

Researchers at Mt. Sinai School of Medicine and New York Medical College have proposed a mechanism to explain why male alcoholics with or without liver disease often show certain hypogonadal features. These include impaired sperm formation, a decrease in testicular size, and impotence and decreased libido. These NIAMDD grantees fed a liquid, nutritionally adequate diet to two groups of rats. The diets were identical except that alcohol was substituted for carbohydrate in one of the groups. After 24 days on these diets, an enzyme (5-testosterone reductase) was greatly increased in the liver of the rats fed the alcohol diet when compared with the controls. In terms of its impact on the body, this means that testosterone is being broken down more quickly by the liver in the alcoholic rat and therefore less testosterone would be abailable to maintain the function of the gonads.

To extend the findings of animal experiments to man, five male

volunteers were studied before starting the alcohol diet and then after 4 weeks on a nutritionally adequate diet that contained 42% of the calories as alcohol. The five volunteers studied showed increases of testosterone reductase activity that were even greater than shown in the rat studies. These changes may play a role in the altered androgenic activity of the chronic alcoholic.

4. Gallstone Formation in Obesity

Obesity has long been suspected of contributing to gallstone disease but epidemiological studies have been inconclusive. Nevertheless, many studies have shown that the two are correlated. Now a biochemical approach is reported which follows the lead opened by the understanding of the physicochemical basis of gallstone formation. That is, the presence of a bile supersaturated with cholesterol relative to the solubilizing agents in the bile (bile acids and phospholipids) favors gallstone formation. A grantee at U. Calif., San Diego, and an NIH scientist working at Phoenix, have shown obese healthy subjects have a significantly greater amount of cholesterol in their bile than a matched group of non-obese subjects. When the obese subjects were studied after a period of weight reduction in which a constant weight had been reestablished at a new lower level, the cholesterol saturation of the bile decreased. An unexpected observation was the fact that during the transitional period of active weight reduction, the cholesterol saturation of the bile did not decrease but actually increased in 6 out of 10 obese subjects. This later effect may be due to mobilization of cholesterol from adipose tissue pools for secretion into the bile.

5. Diagnosis of Acute Cholecystitis

The differential diagnosis of acute abdominal pain still remains a difficult clinical problem. One of the common causes of such distress is cholecystitis, a condition in which the cystic duct becomes obstructed, usually by small gallstones. This duct leads the bile into the gallbaldder from the liver where it is synthesized and then back out again into the intestine when the gallbladder contracts. The two procedures currently used, oral cholecystography and intravenous cholangiography, both can rule out the possibility of complete cystic duct obstruction and therefore acute cholecystitis if the gallbladder is opacified. However, the disorder can not be definitively said to exist if the gallbaldder does not opacify. A group of NIAMDD supported radiologists at Johns Hopkins have developed an adjunct to these methods which appears highly encouraging in the small series of patients tested. This is a procedure in which the gallbladder is first stimulated to empty by the i.v. injection of cholecystokinin. A radiolabeled biliary marker (^{131}I rose bengal or ^{99m}TC dihydrothioctic acid) is injected and the accumulation of radioactivity in the liver and gallbladder regions monitored by external gamma emission between 60-90

minutes after injection of the tracer. This test was able to diagnose cholecystitis in 10 of 39 patients, 9 of whom were confirmed at operation. By the older tests, five of the same 10 patients could not be adequately diagnosed.

B. Contracts

National Cooperative Gallstone Study NO1-AM-3-2216
Cedars-Sinai Medical Center, Los Angeles, Calif.
$7,215,540 for 5 year period

The Contractor, acting as the Coordinating Center, was given the following objectives to accomplish; considered Phase I and Phase II:

I (a) Organize appropriate committees, prepare a protocol and procedures manual: (b) select treatment centers, drug supplier, animal toxicology lab, histology lab, serum and bile chemistry labs by the competitive route and (c) supply the necessary data to obtain FDA permission for the clinical trial on the investigational drug.

II Coordinate and be responsible for the execution of a clinical trial to determine the efficacy and safety of chenodeoxycholic acid (an experimental drug) in the dissolution of gallstones. Also to select, competitively, research studies which will contribute fundamental information on mechanisms of gallstone formation and dissolution of gallstones on the patients in the clinical trial.

The Contractor finished Phase I approximately two years after initiation of the contract and entered the clinical phase, Phase II, in September 1975. Currently, the 10 treatment centers are recruiting, evaluating, testing and starting to randomize appropriate gallstone patients into one of the treatment regimens (375mg or 750mg chenodeoxycholic acid) on a double-blind basis. The safety of the drug is being carefully followed by multiple liver function tests as well as liver biopsies to monitor liver histology. After 100 patients have been randomized and if no adverse liver histology is observed during that time, the main 900 patient study involving the same two dose regimens plus a placebo group will be randomized and followed for two years on the drug regimen.

The significance of this study is the potential for using this drug, if it is proved to be safe and effective on a long-term basis, in patients with gallstone disease who are unable or unwilling to undergo the usual surgical removal of the stones.

The proposed course of the contract is to procede according to schedule. If any significant toxicity occurs, the contract will be terminated with appropriate phase-out to allow follow-up of patients already in the study and to allow compilation of results.

C. Program Projects and Centers

1. Program Projects

Three large multidisciplinary research efforts devoted to the study of liver disease have been reviewed, approved and funded. The projects and their directors are:

a. Liver Metabolism 1 PO1 AM18976-01
   Virginia Commonwealth University
   Richmond, Va.
   Program Director: Dr. Harold J. Fallon

b. Studies of Liver Injury 1 PO1 AM19124-01
   Massachusetts General Hospital
   Boston, Mass.
   Program Director: Dr. Elliot Alpert

c. Program-project in Liver Diseases: 1 PO1 AM 19329-01
   University of Texas
   Dallas, Texas
   Program Director: Dr. Burton Combes

2. Centers

An interdisciplinary liver research program, involving 28 investigators, 18 associate investigators and a number of post-doctoral research associates are involved in this program. There are 24 approved projects encompassing the areas of (1) hepatitis and cirrhosis, (2) hepatic drug metabolism, (3) hepatocyte structure and function, (4) hepatic secretion, cholestasis and bile acid metabolism, (5) normal and abnormal hepatic development and differentiation and,(6) metabolism of the bile pigments and porphyrins in health and disease.

The project is located at: University of California
San Francisco, Calif
Program Director: Dr. Rudi Schmid

ANNUAL REPORT - FY 76
NUTRITION PROGRAM AREA

The Nutrition Program of NIAMDD-EP with a total budget for FY 76 of 8.902 million dollars, has continued to support research and training in basic and clinical nutrition, especially in those areas where additional emphasis is needed to achieve the goal of improved health and longevity. Although the amount of monies available to the Nutrition Program during FY 76 was comparable to that in FY 75, the number of research grants funded continued to drop, to 147, as compared to 209 grants funded in FY 67. Approximately an equal amount of nutrition research has been supported by other extramural program areas of NIAMDD (particularly Metabolism, Hematology, Diabetes, and Endocrinology) where nutrition is a secondary but significant component. NIAMDD supports approximately 1/3 of nutrition research represented in the different institutes of NIH, and has a greater responsibility for training in nutrition through fellowships and training grants than all other institutes.

The widespread prevalence of nutrition-related diseases such as atherosclerosis, diabetes, obesity, osteoperosis, alcoholism, etc., as well as the existence of malnutrition in many segments of the population, clearly document the need for more efforts in nutrition research. The greatest promise for solutions to these problems lies in fundamental studies, such as those on absorption, metabolism, and mechanisms of action of nutritional factors, the biological control of such processes and the identification of yet unknown essential nutrients and their metabolites. Recent technological advances such as mass spectrometry, high pressure liquid chromatography and radioimmunoassay should permit rapid progress in basic biomedical nutrition studies.

The NIAMDD Nutrition Program is giving special emphasis to the following research areas: obesity, parenteral nutrition, nutritional anemias, trace element nutrition, dietary fiber, mechanism of action of nutrients, nutrient interactions and factors that modify nutrient requirements.

Trends in Experimental Research

1. OBESITY - Obesity has been given special priority for 2 years. In May 1974 this institute funded the first NIH obesity center at St. Luke's Hospital, NYC, in an effort to expidite research in this important area. This center has been quite effective in bringing together investigators who share an interest in obesity research, through monthly meetings, library facilities, consulting and coordinating activities.

Their studies on eating behavior reveal that obese patients will modify there food intake as needed to maintain correspondingly high energy intakes when lower energy foods are offered, suggesting that obese persons may regulate their physiological appetite around a higher "set point" of body fat content. This center also has completed the first human study where body composition changes were followed in subjects fed (1) no food, (2) a low carbohydrate ketogenic 800 calorie diet and (3) an 800 calorie

"mixed" diet containing normal proportions of carbohydrates and fats. As was expected the low carbohydrate ketogenic diet and short term starvation both produced more rapid weight loss than did the "mixed" diet, but the increased loss was due primarily to loss of body water associated with the breakdown of body protein. The ketogenic diet had no metabolic advantage - it did not increase the rate at which fat was metabolized, nor did it spare body protein. The Center is developing a demonstration model program for clinical management of the obese patient.

Expression of interest to fund obesity centers led to a noticeable increase in related research applications. Studies by McHugh at Johns Hopkins with monkeys have demonstrated the capacity for control of caloric intake in primates. Satiety was expressed as a graded response to several mechanisms, as gastric distension, caloric regulation and energy storage in an integrated fashion. Koopmans, Columbia University, has obtained results which indicate that the nerves of the stomach and upper intestines are not responsible for hunger satiety. In this connection, Gibbs, Cornell Medical College, has obtained evidence in the rat which supports the hypothesis that the intestinal hormone, cholecystokinin may serve as a physiological satiety signal. Currently, the nutrition program is funding 29 research grants on appetite, feeding behavior, and obesity at a level of approximately 1.7 million (1.22 million direct costs). This amount should be increased to 2.1, 2.3 and 2.5 million during the next three years.

2. PARENTERAL NUTRITION - Despite the need for the wide application of parenteral nutrition in modern medicine, many unanswered questions still remain about the nutritional components of parenteral fluids, especially with regard to the lipids and trace elements. This area has been announced as an emphasis area and four research grants amounting to approximately 380 thousand dollars are being supported. These include studies on trace metal and fatty acid requirements of patients.

The lack of more complete information on nutrient requirements in general renders the problem of developing adequate safe mixtures for parenteral feeding even more urgent. The need to develop proper mixtures for use in total parenteral nutrition is imperative for the management of contenital anomalies and various disorders of the gastrointestinal tract, renal and hepatic failure, trauma, burns and post-surgical states. The budget for this purpose should be escalated to approximately 0.75 million dollars within three years.

3. TRACE ELEMENTS - Recent research has demonstrated the need for tin, vanadium, fluoride, silicon and nickel for growth and well-being of animals. The role of zinc, chromium and selenium in metabolism of animals and man has been clarified.

K. Schwarz has observed in growth studies with silicon, that a large variety of materials considered to belong to the broad category of "dietary fiber" actually contain very high amounts of silicon. He has suggested that some of the preventive efforts attributed to "dietary fiber" may be due to the silicon content. Zinc therapy also has been found to be effective in Acrodermatitis Enteropathica.

It is likely that trace mineral deficiencies and imbalances may be involved in the cause of chronic, adaptive diseases. To this end, there is a real need for the systematic collection of trace mineral content of various human tissues together with other information necessary for appropriate epidemological analysis.

Iron deficiency anemia is still a widespread public health problem, which lends itself to dietary supplementation Studies are needed to evaluate the health significance of nutritional anemias, with emphasis on effect on work performance and susceptibility to infection. Recent advances in methodologies for determination of iron stores and bioavailability of iron from available foods offer the possibility of conducting a proper field study to measure the effectiveness of dietary iron supplementation.

Research grants in the area of mineral nutrition number 20, at a level of 1.15 million dollars. This should be increased to at least 2.0 million in the next three years.

4. PROTEINS AND AMINO ACIDS - The nutrition program continues to fund 20 research grants dealing with protein and amino acids amounting to approximately 1.15 million dollars. These include studies on amino acid requirements in preschool children and adults, methods of assessing nutritive value of proteins, effect of energy intake on protein utilization and various aspects of protein metabolism. A very encouraging finding deals with the use of keto acids in treatment of uremia. Since the body can make the "dietary essential" amino acids (except for lysine and threonine) from the corresponding keto acid by the addition of amino nitrogen from urea, the use of these 7 keto acids plus lysine and threonine in lieu of dietary protein in patients with impaired kidney function looks most promising. Dr. M. Walser, Johns Hospkins School of Medicine, has found that such treatment results in a reduced nitrogen excretion load and improved kidney function and that it eliminates the need for dialysis in most patients.

5. VITAMINS - Of the 42 grants supporting research on vitamins, 22 dealt with the fat soluble vitamins (A,D,E and K), amounting to 1.26 million, and 20 relate to vitamin C and the B-complex vitamins, with a total of 916 thousand dollars. Research advances are being made in understanding the mechanisms of action of these vitamins in specific metabolic pathways. The recent discovery that the active form of Vitamin D in the body involves its conversion to the 1,25 dihydroxy form, and that the kidney is the site of hydroxylation at the 1 carbon position, offers a solution to osteoporosis in persons with impaired kidney function, including aging women. This results from findings by Dr. Hector DeLuca, University of Wisconsin, concerning the conversion of Vitamin D to its hormone-like active form which affects the removal of calcium from the bones as well as absorption from the lumen of the intestine.

Substantial progress also is being made in the isolation of the protein in human plasma responsible for the transport of Vitamin D and the 25-hydroxyvitamin D. (Goodman, Columbia University).

6. DIETARY FIBER - The recent interests in the possible health significance of dietary fiber in prevention of certain gastrointestinal diseases, ischemic heart disease and even some forms of cancer will require increased research support in this area. At present, little is known about the chemical characteristics of so-called "food fiber", their effects on intestinal microflora and on food transit time. The presence of certain types of fiber on food are known to chelate certain mineral nutrients and bile acids. Food fiber, as it exists in foods, also is known to reduce the digestibility of foods and reduce caloric intake. A workshop has been planned on the role of food fiber in health under the leadership of Dr. Harold Roth (Feb, 1977). It is anticipated that a budget of approximately 1/2 million dollars will be needed in FY 77 for research in this area.

7. LIPIDS - Twelve research grants on dietary fats, fatty acids or lipid absorption and metabolism are being supported in FY 76, amounting to approximately 810 thousand dollars. Of special interest, Hashim, St. Luke's Hospital Center, has found that medium chain triglycerides (MCT) fed to infants have a striking effect in diminution of steatorrhea and creatorrhea as compared to long chain triglycerides. A significant improvement in calcium and magnesium absorption also was observed in infants fed MCT as 80% of the dietary fat.

Malnutrition Panel (US, Japan CMSP)

The Malnutrition Panel of the US-Japan Cooperative Medical Science Program held four panel meetings and one joint meeting with the members of the Japanese Panel during FY 76. In conjunction with the joint meeting held in Berkeley, California, the US and Japan Panels co-sponsored a 3 day symposium on "The Biological and Cultural Sources of Variability in Human Nutrition", which was attended by 678 persons. In addition the US Panel held two workshops - one on "Malnutrition and Infection" and another on "Anemia - Iron Deficiency and Other Causative Factors" to identify research needs in these areas. Another jointly sponsored symposium on "Nutritional Deficiency Secondary to inborn errors of Metabolism" is planned in Sendai, Japan on December 2-3, 1976.

The US Malnutrition Panel has continued to review R22 research grant applications, and at present 13 research grants and 2 contracts are being funded as a part of the US-Japan Malnutrition Program.

Fellowships, Careers and Training

Due to delayed National Research Service Act authority and funding for training, very few new awards have been made. Only two new Research Career Devalopment awards (K04) and one Individual Postdoctoral Fellowship (F32) have actually been awarded at this time (May 1976). Hopefully funds will become available before the end of FY 76 to fund most of the F32's, K04's, K07's and T32'a which have been approved at very good priorities and would help to further the goals of the nutrition program. One new institutional training grant application entitled Clinical Nutrition for Physicians was deferred from June 75 council for a site visit, which took place in July 75. Even though it

was deemed to be a very important nutrition training opportunity and received a most admirable priority score, this application has not as yet been funded because of the delayed authority and funding.

At present there are ten approved Fellowship and Career applications nine of which should be funded in FY 76. Of the seven approved but pending training grant applications (T32), it is hoped that two or more can be started with FY 76 funds.

In spite of the meager number of new applications funded in FY 76, the nutrition program continues to support a variety of "old" and new types of training grants, fellowships and careers. These active continuation grants include: 4 training grants ("old" T01 - 2, "new" T32 - 2); 18 fellowships (F22 - 9, F32 - 9); 10 research career awards (K06 - 3, K04 - 3, K07 - 4).

Following is a brief description of the different career awards. The Career Award (K06) was originally offered in 1961-64 by NIH to enable institutions to support investigators of high competence for the duration of their careers. The award is terminated at time of retirement of the awardee. The Research Career Development Award (K04) is limited to a single five year period for individuals with clear research potentials but requiring additional training and expertise in preparation for careers in independent research.

The Academic Career Development Award (K07) in digestive diseases and nutrition was offered in 1971-72 by NIAMDD to provide an academic position in an institution with a demonstrated need. Need was identified in terms of the academic leadership required for initiation or augmentation of essential activities to strenthen teaching, and research in nutrition and digestive diseases.

At present, no Clinical Investigator Awards (K08) are being funded in the nutrrition program area.

Official Duties

Dr. Gerald F. Combs replaced Dr. Karl E. Mason as Director of the Nutrition Program, NIAMDD-EP on September 1, 1975. Mrs. Marjorie Zukel is Assistant Director. The Nutrition Program Director continues to serve as Program Officer of the Malnutrition Panel of th US-Japan Cooperative Medical Science Program. In addition he serves as a liason member to the Food and Nutrition Board, NRC/NAS, and is a member of the Board of the Society for Nutrition Education. He has also chaired the committee on human nutrition, as a part of the USDA working conference on "Research to Meet US and World Food Needs".

Hematology Program

The Hematology Program of the National Institute of Arthritis, Metabolism, and Digestive Diseases supports fundamental and clinical extramural investigation into the cause, prevention, diagnosis, and treatment of a broad spectrum of hematologic disorders. The emphasis is on metabolic aspects of these disorders, commensurate with the mission of the NIAMDD, since its inception in 1950, to encourage and conduct research on metabolism and metabolic diseases. The following hematologic disorders are representative of the projects supported by the program:

1) genetic anemias, primarily those due to genetic abnormalities of hemoglobin and hemoglobin synthesis (Cooley's anemia, sickle cell anemia).

2) nutritional anemias, including those involving iron, folic acid, and vitamin B_{12} deficiencies.

3) other metabolic disorders related to iron transport and storage, porphyrin metabolism (porphyrias), and enzymatic defects (red cell glucose-6-phosphate dehydrogenase deficiency.)

4) disorders of blood cell production, such as the anemia of chronic renal failure, aplastic anemia, and polycythemia.

5) immunology of bone marrow transplantation and autoimmune hemotologic disease.

These subjects are of prime hematologic interest, but also relate significantly to efforts of programs in this and other Institutes in areas such as chronic renal insufficiency, transplantation, and malignancy. The Hematology Program budget for support of extramural research and training represents approximately 8 per cent of the total NIAMDD extramural budget for FY '75.

Genetic Anemias

Cooley's anemia (thalassemia) and sickle cell anemia are the subject of approximately 23 per cent of the projects supported by this program. Since research related to these anemias falls within the mission of both this program and those of the National Heart and Lung Institute, it is imperative that good communication and coordination of effort exist among the staff of the programs. Projects on both anemias are assigned to the NIAMDD Hematology Program according to their basic metabolic emphasis, including research on regulation and genetics of hemoglobin synthesis, hemoglobin structure and functional relationships, form, function and demise of the sickle or otherwise defective red blood cell, prenatal diagnosis of conditions on the basis of hemoglobin or other metabolic analysis, and metabolic consequenses of the anemias.

Two projects administered by this program are presently funded from the Sickle Cell Disease earmark of the NHLI, while 16 additional grants with

primary emphasis in this disorder are being supported by NIAMDD funds.

a) Identification and characterization of abnormal hemoglobins.
One such project at the University of Texas Medical Branch in Galveston has developed into an important national reference resource, assisting other laboratories to confirm the identity of sample hemoglobins.

b) Mechanism and kinetics of the aggregation of the hemoglobin S molecules.
Initial, intermediate, and final stages of the sol-gel transformation of deoxyhemoglobin S are being studied in order to devise techniques of interfering with the process.

c) Analysis of structure and function relationships in normal and sickle hemoglobins, and attempts to find antisickling agents.
X-ray crystallographic studies as well as other physical techniques are used to determine the role of various components of the hemoglobin molecule in the aggregation process, with the intent of devising techniques to retard or prevent this process, such as through the use of therapeutic drugs.

d) Studies of the regulation of the synthesis of hemoglobins.
Seven active projects are investigating the genetic regulation of the synthesis of fetal hemoglobin and adult hemoglobin in such systems as amphibian metamorphosis, hamster erythroid cell cultures, and conditions of hereditary persistence of fetal hemoglobins in humans. Since the severity of the clinical manifestations of sickle cell anemia is related in part to the concentration of the defective hemoglobin (hemoglobin S) within the red cell, sickle cell anemia patients who continue to synthesize high concentrations of fetal hemoglobin may be relatively asymptomatic. The hope is to find feasible means to augment the synthesis of fetal hemoglobin and to modulate hemoglobin S synthesis.

e) Studies relating to the injury to the red cell membrane in sickle cell anemia.
Work at the University of Minnesota has shown that purposely loading hemoglobin SS red cells with excessive calcium induces irreversible sickling. The possibility exists that the myosin-like membrane protein spectrin is altered by excessive calcium, deforming the normal red cell shape. Related work by this group has shown correlation of red cell calcium abnormalities with the ischemia typical of the disease, suggesting that therapy must reduce red cell calcium permeability and content to be effective.

A study with proven clinical utility by Dr. Yuet Wai Kan at the University of California in San Francisco and co-workers at Harvard and at Yale has resulted in successful diagnosis of sickle cell anemia in a fetus in utero. Previous diagnoses of fetuses at risk for Cooley's anemia have also been performed.

Many of the other studies relating to sickle cell anemia bear directly on Cooley's anemia, which also is caused by a defect in hemoglobin synthesis. For example, the ability to modulate regulation of hemoglobin synthesis in favor of the fetal form could result in alleviation of Cooley's anemia. In addition, twenty-two grants amounting to more than 1.3 million dollars of FY '75 funds were directed specifically to solving aspects of this disease which affects individuals of Mediterranean and Far Eastern ancestry.

Projects related to Cooley's anemia, in addition to basic hemoglobin research, deal with genetics and distribution of defective globins, metabolic effect of the disease, characterization of the consequences of therapy and design of new and more effective therapeutic drugs.

An important aspect of this research is the study of globin synthesis in families with various types of Cooley's anemia (thalassemia). One such study has shown that excess α chain is probably a major cause of the severe anemia of homozygous thalassemia. That some homozygotes whose anemia is mild appear to produce as much α chain as those whose anemia is severe suggests that the mechanism of α chain elimination may be important in determining the severity of the disease. Another study has shown that, at least in rabbit bone marrow, β chain synthesis is regulated in part by α chains. The esistance of such a mechanism in α thalassemia could account for the reduced percentage of the abnormal β -chain in certain forms of the disease.

Patients with the severe anemia associated with certain forms of Cooley's anemia are often treated with regular transfusions. An important toxic side effect is the resulting iron overload. Thus studies of iron storage and mobilization in normal and iron overload conditions have important implications for therapy. Some of the research involves the consideration of the effect of chelators on iron-loaded duodenal cells and other tissues with the interest of determining how these chelators act on the various iron compartments. Other projects are aimed at determining the theoretical requirements for the development of a suitable drug for treatment of iron-load conditions, and at finding and testing such drugs.

Nutritional Anemias

Nearly all of NIH-supported projects investigating iron metabolism and iron deficiency anemia are administered by this program. Some of these studies bear on understanding iron absorption and transport in an effort to improve iron nutrition in the human population. Phosphoproteins from egg yolk and milk have been examined as possible causative factors in the poor absorption of naturally occurring and supplemental iron from eggs and milk. It was concluded that milk casein, at least, actually improves the intestinal absorption of the ferric nitrilotriacetate chelate, used to donate iron (III) specifically to protein phosphorylserine groups. Milk, therefore, if fortified with suitable iron chelates, would make an excellent vehicle for iron

supplementation of a target population.

A system related to iron metabolism, intestinal cell oxidative drug metabolism, may be an important detoxification mechanism. The enzyme systems involved, and their component cytochrome P-450 have been found to be located primarily in the absorptive cells of the intestinal mucosa. The synthetic process leading to cytochrome P-450 requires iron derived from absorbed luminal iron; body iron is not available for formation of this cytochrome in intestinal cells. This finding has important implications for individuals with an iron-poor diet.

The absorption of vitamin B_{12} across the intestinal mucosa is a complex process of a critical nature in such conditions as pernicious anemia, but elucidation could have implications for understanding absorption of other nutrients. Intrinsic factor, which mediates B_{12} uptake, is bound by a protein (receptor protein) on the ileal epithelial mucosa. Purified receptor protein is being characterized and its properties determined. Focus is also being applied to the release of B_{12} from its attachment to intrinsic factor, particularly to whether releasing activity is subcellular or on the surface of microvillus membranes. Resolution of this point would help explain the mechanism for congenital deficiency of B_{12} absorption. Plasma transcobolamine II, which facilitates the uptake of vitamin B_{12} in a number of cells has been isolated in homogeneous form and radioatively labelled, and its role and fate during the process of B_{12} binding to cell surfaces, and intracellular migration is under study, as are the tissue distribution, role, and mechanisms of folic acid binding proteins. Here the emphasis is on the potential clinical uses of measurement of these proteins in various conditions including uremia, lithium induced granulocytosis, and myeloproliferative disorders.

Metabolic Disorders related to iron, porphyrins, and enzymatic defects

Significant progress continues in the overall metabolism and transport of iron. Normal cellular iron metabolism is essential to human health. Although pathological accumulation of iron in cells occurs under a wide variety of conditions, very little is known about the factors leading to it. It occurs in chronic alcoholism, Kwashiorkor, patients who have received large numbers of blood transfusions, such as Cooley's anemia patients, and those with idiopathic hemochromatosis.

The central role of iron-binding proteins in mediating the over-all iron metabolism of the body is becoming increasingly well documented. Normally, iron is stored mainly as ferritin and the stores are called upon when there is anemia. More needs to be learned about the manner in which these stores are established, depleted, or overloaded. Important in defining the factors involved in the uptake and release of iron by various key tissues throughout the body was the significant observation that tissues - specifically rat intestine - have the ability to deliver iron selectively to one of the two binding sites of the transferrin molecule, a crucial iron-binding protein. This extends an earlier confirmation of the Fletcher-Huehns hypothesis of selective release of iron to tissues that recognize iron on one of the two binding sites, and is considered essential in establishing the biological

significance of the cycle of selective tissue uptake and delivery of iron by transferrin.

Several projects are attempting to describe the mechanisms by which iron is transported within the developing red blood cell, for use in formation of hemoglobin. There are two schools of thought on this subject. Some workers in Australia and Czechoslovakia are proponents of the concept that serum transferrin actually enters the developing cell and carries iron to sites of utilization. A more widely held hypothesis is that serum transferrin relinquishes iron to specific receptors on the plasma membrane of such cells, with subsequent transmembrane transport to cytoplasmic transport agents. In accordance with the latter hypothesis, a 6,000 molecular weight iron binding protein has been found in the cytoplasm, and has been suggested as an important cytoplasmic iron transport agent. This molecule is undergoing study with regard to number of binding sites, oxidation state of the iron, and amino acid composition. Functional studies could help resolve this aspect of what is currently a controversial area.

The complex organo-metallic porphyrins are central to the function of hemoglobin and related oxygen-binding proteins. The oxidation states of various porphyrias have now been clearly established, in the process of understanding the relation of metal electrons to porphyrin electrons and their respective roles in redox. The biosynthetic enzymes involved in porphyrin metabolism progressively are being characterized and their structure and specific actions elucidated. The interaction of a porphyrin-binding protein, hemopexin, and various forms of porphyrins has been analysed, and it is suggested that this protein may be instrumental in the tissue distribution and route of excretion of coproporphyrin, since the interaction is strongest with this porphyrin. A unique herd of porphyric cattle at the University of Minnesota is providing a useful animal model for studies, in cooperation with several other laboratories, of manifestations and complications of aberrant porphyrin metabolism which leads to extreme photosensitivity of skin and other symptoms.

Disorders of Blood Cell Production and Destruction

Diminished renal production of the hormone erythropoietin can cause a variety of significant anemias, of which the anemia of chronic renal failure is one. The mechanism and regulation of erythropoietin biosynthesis, still unknown, is under study in primary kidney cell cultures, which have been shown to release appreciable amounts of erythropoietin into the medium. Release may be regulated through hormones or oxygen concentration. A major need is for purification and complete characterization of erythropoietin, and development of a specific highly sensitive assay. Significant progress has been made in the development of a radioimmunoassay for erythropoietin using the lactoperoxidase and Chloramine-T methods, found to be sensitve enough to detect as little as 0.025 milliunits of the International Reference Preparation erythropoietin.

Exposure to hemodialyses which utilize unpurified or reverse-osmosis purified water may produce or markedly worsen uremic hemolysis. The cause appears to be that chloramines, widely used as bactericidal agents in many urban water supplies, produce oxidative denaturation of hemoglobin, resulting in Heinz

body hemolytic anemia. Ascorbic acid added to dialysis baths in physiologic concentration prevents this damage and normalizes red cell survival.

Hemolysis of aging red cells has prompted an evaluation of enzymatic capacities, resulting in evidence that only 3 enzymes of glycolysis are significantly age-dependent (hexakinase, aldolase, and pyruvate kinase). Since aging of red cells is accompanied by a progressive decline in ability to increase basal metabolic rates, senescent cells are incapable of producing a metabolic surge under stress conditions.

The red cell membrane plays a major role in the integrity of the cell, and thus is a candidate for study in the search for other potential causes of hemolytic anemias. Patients with herediatry hemolytic anemia possess many abnormal cells (target cells or stomatocytes) which seem to have membranes which are unusually permeable to calcium. The two to three-fold increase in calcium influx may influence the deformability of the membrane and play a role in cell destruction.

Another project produced evidence of considerably reduced phosphorylation in three membrane proteins in red cells of patients with human herediatry spherocytosis, suggesting that membrane phosphorylation might be the energy dependent process responsible for the control of cell shape and deformability which are both abnormal in that disorder. Membrane kinase activity may be related to the maintenance of normal cell shape, and may also be related to the iron transport disorder in herediatry stomatocytosis.

Many other studies of the red cell membrane are supported by this program, and which bear on a wide variety of disorders. This membrane has found wide use due to its ready availability and ease of purification.

White cell production studies are also supported. In cultures of normal mouse marrow cells grown in diffusion chamber cultures there was a steady-state of granulocyte renewal analogous to granulopoiesis in the normal marrow during later phases of culture. This suggested that white cell development in diffusion chamber cultures is subject to fine regulation by factors intrisic to the cultured cell population. The system is being used to study the role of cell -cell interactions and diffusible substances of normal and unregulated leukemic cells.

A study on the mechanism by which estrogens affect hematopoiesis has shown that injection of various synthetic and naturally-occurring estrogenic steroids into mice results in moderate neutropenia and thrombocytopenia but not anemia. This was associated with increased splenic erythropoiesis and a compensatory decreased rate of erythropoiesis in the osteosclerotic marrow. The mechanism of this compensatory phenomenon is being investigated.

Other studies include experimental drug therapies for patients with bone marrow deficiencies, development of new techniques for the culture of human bone marrow in vitro, and examination of the ultrastructure and functional relationships of leukocytes.

Immunology of Transplantation and of Autoimmune Hematologic Disease

The study of histocompatibility is in a vigorous and expanding phase, with many projects engaged in the elucidation of mechanisms involved in recognition and rejection of foreign grafts of hemopoietic cells, as well as investigations of the relationship of HL-A antigens and disease.

Transplantation of normal bone marrow cells to patients with certain blood diseases such as aplastic anemia and leukemia represents a potentially useful therapy. However, such transplantation is often followed by graft-versus-host reaction and subsequent immune disease. These complicating immune reactions may also be due to reaction of the recipient's cells to those of the graft. The standard technique used to prevent recipient reactivity, whole body irradiation of the host, has been shown in experimental animals to be ineffective in allowing hemopoietic cells to grow. For the study of this unusual, little-understood reaction, an *in vitro* model is of importance, both for understanding the immune mechanism as well as for matching prospective donors and recipients. Recently one project has developed such a model. Spleen cells from a hybrid strain of mouse were cultured and sensitized against irradiated cells from the inbred parent lines with which the hybrids were produced. The cultured spleen cells showed the capacity to selectively react against the parental cells, with immune reactions similar to those in hybrid rejection of parental bone marrow *in vivo*.

Clinical evaluation of a new drug, Oxisuran, has shown potential use for suppression of graft rejection. Oxisuran appeared non-cytotoxic in all patients tested (9), and does not interfere with the regenerating graft. Its action is to block the start of new immunologic reactions, without restricting normal immunologic capability. Thus the drug has the advantages of inhibiting both graft rejection and secondary disease, without interfering with the growth of needed red blood cells and other marrow cell elements. Also, since the majority of transplant deaths occur from severe infection with microorganisms normally present, Oxisuran therapy may prevent such opportunistic infections by preserving the immunologic capability of the recipient to respond against them.

Several studies are proceeding on the genetics and chromosome mapping, development, and physical arrangement of antigens on the plasma membrane, particularly those of the lymphocytes, HL-A antigens. Studies of the association of HL-A antigens with disease susceptibility and immune response have shown numerous correlations. For example, an analysis of antigen frequency in psoriatic arthritis patients has shown that the high incidence of W27 antigen, previously observed in ankylosing spondylitis, and the high evidence of W17 present in psoriasis are increased in those patients. The two antigens are rather infrequently present in the same patient with psoriatic arthritis. It is postulated, therefore, that the antigens associated with psoriasis and spondylitis may exist in these patients as two different entities.

Methods by which lymphocytes can be stored and sent through the regular mails have been developed. In appropriate tissue culture media. lymphocytes can be kept viable for as long as three weeks, with an average viability

of 1 per cent per day. This technical advance will improve the ease of testing HL-A antigens and in cross-checking of specificities between laboratories.

Splenic and marrow cells have been shown to produce a platelet - specific antibody, confirming the immunologic nature of immune thrombocytopenia. In the case of the spleen, estimations have been made of the specific material produced. Future studies are planned to allow better evaluation of the marrow's contribution and the effect of immunosuppressant agents on antibody production.

Other projects deal with characterization of antibodies of infectious mononucleosis, characterization of the process of complement synthesis, and the genetic control of normal and abnormal immunological response, with particular emphasis on the pathogenesis of autoimmunity and plasma cell neoplasia.

Training

The number of institutional training grants supported in this program fell to 16 in Fy '75, as these grants continue to be phased out. Thirty-four trainees were on blood in these programs. Six new Institutional Research Service Awards were made, and 6 postdoctoral candidates were supported by these. Four Individual National Research Service Awards were made, with a total of 13 fellows supported by this and older fellowship programs.

Hematology Program Area

Iron Chelators -- Contract Program

Introduction

This program was initiated in July of 1974 with the award of four contracts for the development and testing of safe, more effective compounds to remove pathological stores of iron from the body, particularly those resulting from repeated transfusions as in the treatment of Cooley's anemia. Two of the contractors are synthesizing new iron chelators based on chemical and pharmacological principles and characterizing these agents with physical-chemical procedures. One of them is concentrating on multidentate ligands derived primarily from the phenols, carboxylic acids and tropolones while the other is concentrating on hydroxamic acid derivatives. A third contractor is isolating iron-binding compounds from bacterial sources. The compounds produced by these efforts and, in addition, those submitted by others not funded by the program, are then screened in a transfused mouse assay developed by the fourth contractor. The compounds are screened for their ability to promote iron depletion in the liver and spleen and iron excretion and for their general toxicity. In a cooperative activity, not supported by the Iron Chelation Program, selected compounds are also being tested in a tissue culture system for their ability to bind iron. Through appropriate feedback from the testing phase of the program, the contractors who are synthesizing compounds proceed with attempts to chemically modify selected candidate compounds to improve such properties as solubility and toxicity.

A fifth project was subsequently added to the program in which the contractor is conducting a pilot clinical study in Cooley's anemia patients with transfusion hemosiderosis of a compound, 2,3-dihydroxybenzoic acid (2,3-DHB), which he had found to be promising in early studies.

Current Progress and Future Direction

Forty-one compounds have been through initial screening so far. Of these, thirty-four were prepared by the program's contractors and seven came from other sources. Preliminary studies show that, at least at the dose levels used, overt toxicity is not a serious problem. Five compounds have exhibited some degree of efficacy in removing iron and will be studied further with more extensive pharmacologic methods. Some attempts will be made in laboratory animals to evaluate promising new compounds given in combination with deferoxamine mesylate (the chelating drug presently in use), inasmuch as there is some speculation that different pools of iron may be susceptible to various compounds.

DIABETES PROGRAM

During the past year the Diabetes Program of the NIAMDD (along with diabetes-related areas of other Institutes, notably NHLBI and NEI) have been highlighted by the submission to the Congress of the Long Range Plan to Combat Diabetes by the National Commission on Diabetes. The recommendations contained therein with particular challenge to the NIAMDD were:

- To increase its support for research efforts in basic endocrinology, metabolism, and nutrition, and apply the results of such research to the study of diabetic defects and the causes, prevention, and cure for diabetes.

- To increase its support for research efforts in nutrition relating to diabetes and to obesity in persons with diabetes, and increase the number of its Obesity Centers to three to encourage and to train manpower in this area.

- To establish an ad hoc advisory panel on transplantation and artificial devices and, commensurate with the panel's recommendations, increase support of research efforts in transplantation of the pancreas or islet cells and development of artificial devices.

- To increase its support for research efforts in diabetic microangiopathy.

- To initiate and support (with the NHLBI) a five-year study to assess the effect of treatment for juvenile-onset diabetes on the development of micro- and macrovascular complications.

- To expand its ongoing intramural research program related to diabetes.

- To expand its training fellowships and special awards in the area of diabetes-related studies.

- To review all available research information resources in order to develop a system to provide a complete and up-to-date source of information on ongoing and proposed research relating to diabetes.

- To establish, where appropriate to the NIAMDD mission, research resource facilities to maintain research materials and animal models, and provide economical and efficient services necessary to meet the needs of diabetes research.

- To be alert and receptive to the sponsorship of evaluation studies pertaining to diabetes treatment and education as part of the NIH's competitively awarded research programs.

- To sponsor interdisciplinary workshops, symposia, and conferences on diabetes and its complications to encourage cross-fertilization of ideas and cooperative research efforts.

- To work on the implementation of the program for Diabetes and Research Training Centers.

A major area of emphasis in the program has been and will continue to be research efforts of a basic nature with the goals of elucidating the metabolic defects of diabetes and application of such studies to the causes, prevention, and cure for diabetes. These studies, representing a multifaceted approach from the molecular and cellular level all the way through clinical studies, presently include structure-function studies of insulin and glucagon, as well as of related peptides and enzymes, studies of the mode of action of insulin and glucagon including the mechanisms of release, secretion, and biosynthesis. Further elucidation of the role and potential usefulness of somatostatin, which inhibits secretion of insulin and glucagon (as well as a number of other hormones) have continued to further elucidate metabolic interrelationships relative to the pathophysiology of diabetes. In concert with the studies on hormones, are studies on carbohydrate metabolism and gluconeogenesis, lipid and protein metabolism, and nutritional interrelationships, including obesity. (Studies on obesity with importance to diabetes are also supported by the Nutrition Program).

Further light has been shed during the past year on a possible cause of diabetes in studies pointing up the role of virus infections prior to the onset of the disorder. Certain HLA antigen tissue types have also been found to occur with greater prevalence in the diabetic population than in the normal. These initial investigations bring out an important new lead which may shed further light on the genetic basis of diabetes.

The question of the treatment for patients who presently have the disease

must be accorded prime relevance while we are still searching for cure or prevention. One approach to treament which is receiving a considerable amount of attention is the possibility of transplantation of the islet cells of the pancreas, since whole organ transplantation has not met with much success, primarily due to the complications induced by the presence of the exocrine portion of the pancreas. Research supported in this area by the Diabetes Program has increased during the past year. Progress has been made in methodology for separation of the islet cells, in culturing islet cells, and in the study of the physiology of islet cells.

A second approach is the continued interest and research in the development of an artifical device to regulate blood glucose. While this program does not presently support any research in this area, plans are well underway to initiate a contract program to facilitate the development of such device(s).

A particularly important question to be answered is whether or not better control of blood glucose, whether by transplantation, use of a device, or other means, will present the complications of diabetes. The study (with NHLBI) suggested in the recommendations is very important in this regard and would also contribute valuable information needed for appropriate design of any artificial device.

The use of oral antidiabetic agents, brought to the forefront a number of years ago by the findings of the University Group Diabetes Program (UGDP) that patients using them suffered increased cardiovascular mortality, continues to be a contraversial subject. This large clinical trial is now being concluded and additional analysis of the data should yield further important information, not only as relates to the oral drugs, but also in regard to the natural history of diabetes. Other studies relating to the microangiopathy of diabetes will also be contributory to the natural history, as well as to the elucidation of the mechanism of the development of the vascular changes leading to the complications of this disorder.

One of the needs identified by the Commission related to training of additional manpower. This is a more difficult problem, because of the vagaries of legislative authorities and funding.

The intramural and information activities relating to diabetes are covered in other areas of this report.

The program has been considering the most efficient and appropriate ways to increase the program emphasis on animal models. Fostering better and increased utilization of given animals is being entertained.

The Diabetes Program currently funds five Diabetes-Endocrinology Research Centers. The Diabetes Research and Training Centers, authorized by legislation (PL 93-354) and contingent upon recommendations of the Commission, are expected to contain components of health professional continuing education and research into evaluation of patient education. Guidelines and regulations are being prepared for these centers, so that if funding resources are available, it would be expected that applications could be received and reviewed during FY 77. Several workshops have recently been sponsored by the NIAMDD, with the basic purpose of exploring areas of potential support from agencies such as NIMH, HRA, and HSA for certain components of these centers.

The Diabetes Mellitues Coordinating Committee (DMCC) has continued their meetings on a bimonthly basis. A report to the Director, NIH was published in December covering FY 74 diabetes-related activities of Federal agencies. Compilation with a more defined analysis and including potential diabetes-related areas covering FY 75 is expected to be published during the summer. The DMCC is currently making an in-depth survey (using a discreet subject -- blindness from diabetic retinopathy -- as a model) of existing, potential, and needed interagency interaction in order to respond most effectively in both services and research. From the exercise with this model it is hoped to extend the activity to other facets of diabetes and its complications. The Diabetes Program Director, NIAMDD-EP serves as Executive Secretary for the DMCC, and also served in this capacity for the National Commission on Diabetes.

Endocrinology Program

The Endocrinology Program supports basic and clinical research on the traditionally accepted endocrine organs. In addition, research is supported on substances which, while not considered hormonal in the usual sense, modify hormone action or are modified by hormones. A major portion of our current research effort is expended in studies on the mode of action of hormones, studies on the *in vitro* synthesis and biosynthesis of hormones, and research on the neuro-endocrine system.

Pineal Gland

The mammalian pineal gland is an endocrine organ which has no direct connection to the central nervous system and whose metabolism is controlled by environmental lighting via an indirect pathway involving peripheral sympathetic nerves. When the sympathetic nerves are stimulated, they release a hormone known as melatonin. It is known to act via the brain and perhaps to act directly to suppress the rate of gonadal maturation and to interfere with the subsequent gonadal function and cyclicity. It may also affect other endocrine organs.

Work supported by the Endocrinology Program has shown that growth and growth hormone are inhibited in rats by blinding and by anosmia; i.e., removing the sense of smell, and that the pineal gland plays an important role in this response. It has been shown that there is an anti-gonadotropin substance from the pineal, melatonin, which can actually inhibit the gonadal atrophy that blinding causes in hamsters. This research has shown that blind and anosmic albino rats do not undergo gonadal involution, if they receive melatonin implants, whereas gonadal atrophy is not prevented in the presence of beeswax implants. Melatonin implants do not reverse the subnormal growth of these rats, however. These results seem to show that the pineal is capable of secreting a potent anti-growth and anti-growth hormone substance. There is still a question whether it is similar to the hypothalamic hormone, SRIF (somatomedin-release-inhibiting factor).

Pituitary Gland

Several stimulating and releasing hormones affect the pituitary gland. Other factors are being studied to see what their role is. One of the grantees is doing studies on prostaglandins found in the blood to see what effect, if any, they have on pituitary function. Several lines of evidence have been developed that endogenous prostaglandins are required for normal thyroid-stimulating hormone secretion in response to either TRH or removal of the feedback inhibition by T_3 and T_4. From work such as this, the researchers have decided that endogenous prostaglandins are involved in regulating TSH and ACTH secretion. In addition, drugs such as indomethacin and aspirin are known to have an effect on prostaglandins and, therefore, the researchers conclude that there is a possibility that there may be an endocrine imbalance because of the wide use of these drugs.

Other studies have been made concerning the hormone LH, or luteinizing hormone from the pituitary. It has been demonstrated that LH circulates in at least two biologically active forms. One of these forms is combined with serum proteins and the other appears to be the same as that produced by the pituitary gland itself. The bound form has a much longer circulatory half-life than the unbound. One of the mechanisms for controlling the concentration of LH may be that it is taken up by cells of the liver and kidneys where it is degraded by lysosomes within the cells. Cells of the testes are able to take luteinizing hormone and degrade it to its constituent amino acids and peptides. It is felt that this hormone uptake by these target tissues may be important in terms of elucidating hormone metabolism.

There is a pathological condition which occurs occasionally following parturition which is known as galactorrhea-amenorrhea syndrome, in which the woman continues to lactate and cannot become pregnant again. Several studies have been made on this syndrome, showing that high doses of pyrodoxin (or vitamin B6) are effective in decreasing the prolactin level of these women so that they no longer lactate and can resume normal menses. Once they stop taking pyrodoxin, however, the condition returns. Another researcher has shown that the drug bromergocryptine will suppress this condition by suppressing prolactin secretion and normal menses are restored. The same researcher indicates that the same drug may be useful in the management of prostatic carcinoma, breast carcinoma, medullary thyroid carcinoma and impotence and he is planning studies along these lines.

Acromegaly has long been considered a disease caused by hyper-secretion of growth hormone from an acidophilic pituitary tumor. However, some recent experiments in animals suggest that the hypothalamic neurohumor growth hormone releasing factor (GH-RF) regulates growth hormone secretion by stimulating the adenohypophyseal acidophils. In addition, plasma from acromegalic patients has been reported to stimulate secretion of growth hormone by pituitary tissue in vitro to a greater extent than plasma from normal individuals. All of these pieces of evidence would indicate that the acromegaly is caused by release of a hypothalamic hormone which in turn stimulates the pituitary to secrete more growth hormone. One researcher has studied patients having a pituitary adenoma which seems to cause acromegaly. By selectively removing only the diseased tissue from the pituitary, he has shown that the acromegaly can be stopped and that the other endocrine function appears to go on normally. On the basis of these results, this researcher feels that acromegaly is indeed caused by a tumor in the pituitary and not by increased hypothalamic hormone secretion.

Endocrine Studies of the Hypothalamus

The hypothalamus is a portion of the brain, yet it releases chemical substances which go via the blood to other endocrine organs, most notably, the pituitary. One of these hormones is somatostatin. This hypothalamic hormone has wide-spread effects on endocrine secretion including the suppression of glucagon, insulin and gastrin, as well as in-

hibition of growth hormone secretion. Somatostatin is thought to be a tetra-deca peptide and the structure of it has now been characterized as a hairpin loop. With this model, it is considered that studies on analogue synthesis can now be done. Other investigators have already made some progress in the development of superactive analogues of somatostatin with the object of making these analogues with a very long active lifetime. Studies of this type show that a selective enhancement of certain of the activities of the hormone can be made, such as increasing an inhibition of glucagon and gastric acid secretion which would be particularly useful for the treatment of diabetes mellitus and gastric ulcers. However, these studies still have a long way to go. It is felt that the development of such analogues may be useful in producing substances that will inhibit certain activities of the normal hormone while increasing other activities of the hormone. Studies on LH-releasing factor are being made to determine the localization of the hypothalamic areas which produce these hormones, the physiological significance of the releasing factors and the role of prostaglandins and cyclonucleotides in releasing hypothalamic hormones. All of these studies are important because they may be used for fertility control. For example, there seems to be little doubt that LRH will be useful in inducing ovulation and it is thought that perhaps analogues of this hormone may be used as an antifertility agent.

Pituitary growth hormone does not seem to act in humans or animals unless there is an additional factor which is now known as somatomedin. This hormone appears to be produced in the liver. Studies have been performed to try to discover the hormonal regulation of somatomedin release by the liver. It has been shown that livers from animals that have been hypophysectomized release less bioassayable somatomedin than does normal rat liver. If the hypophysectomized rats are treated with growth hormone, then the liver increases the amount of bioassayable somatomedin. In addition to growth hormone, clinical observations have led investigators to suspect that insulin also may increase somatomedin release. Studies in rats tend to support this conclusion.

Thyroid Gland

A series of studies in rats have been developed in which hypothalamopituitary, thyroidal and gonadal function of adult rats, treated during a critical neonatal period with various experimental procedures, have been made. In addition to studying the effects of these treatments on the rats as they become adults, the untreated offspring of these rats are also being studied. As a result of these studies, the researchers conclude that experiments with neonatal hyper- and hypothyroidism and those with neonatal administration of substances such as neurotransmitters, alcohol and morphine, all raise the possibility that intra-uterine exposure of the human fetus to these and other substances as well as severe hypothyroidism might result in delayed and life-long alterations in neuroendocrine function. The experimental results add emphasis to the current conservatism regarding the administration of drugs to pregnant and lactating women. Recent studies have shown that similar neonatal

treatment of males also produces abnormalities in their offspring. These changes seem to persist into future generations through both male and female.

A study has been made showing that starvation alters circulating thyroid hormone concentrations in normal men. Serum concentrations of T_3, T_4 and TSH were measured periodically in subjects that were in a fasting condition. The results of these studies are that they have established that a decrease in serum T_3 concentration was a consistent feature of the hormonal response pattern to fasting in both men and women. There was not a significant alteration in circulating T_4 and TSH. The studies indicate that a decreased peripheral conversion of T_4 to T_3 is probably the most likely mechanism responsible for this decrease in serum T_3.

A rather fascinating study has been performed on the kinetics of thyroid hormone, using first some computer models and then finally checking the models in sheep. The model system was set up as a 6-pool structure representing T_3 in plasma, T_3 in liver, kidney, gut, heart and lung which are considered fast tissues and T_3 in skeletal muscle, skin and brain which are considered slow tissues. T_4 was also considered in these same three lumped tissue and plasma pools. By making studies in sheep in these pools, the following results were obtained: Plasma contains 40% of the total T_4. The fast tissue contains 14% of the T_4 and the slow tissue has 47%. By contrast, 12-20% of total T_3 is distributed in plasma, 8-18% is in fast tissue and 62-80% is in slow tissue. In euthyroid sheep, it was found that 40-52% of the T_3 was generated from 11-17% of T_4. However, in hypothyroid sheep, there is no conversion of T_4 to T_3, but hypothyroid sheep do secrete as much T_3 and 40% less T_4 than do normal euthyroid sheep. These results suggest to the researchers that (1) metabolically, T_3 is the more important thyroid hormone as evidenced by the apparent compensatory increase in T_3 glandular secretion relative to T_4 secretion in hypothyroid animals, and (2) that the fast tissues which would be mostly liver, are far more active than other tissues in T_3 metabolism, while T_4 metabolism is essentially unaltered in the "sick" animal. Whereas these statistics are interesting, it is pointed out that the metabolism of thyroxin in sheep is somewhat different from that in humans, since about twice as much T_3 comes from T_4 in humans as in the sheep and about twice as much T_4 is converted to T_3 in humans.

Parathyroid Gland

One researcher has developed a dog model suitable for the *in vivo* study of parathyroid hormone secretion. Injections are made into the thyroid artery and samples obtained from the thyroid veins. Infusion of vitamin D metabolite, $1,25(OH)_2D_3$ consistently stimulates PTH secretion in this model. On the contrary, $24,25-(OH)_2D_3$ infusion into the thyroid artery results in prompt and complete suppression of PTH secretion. These researchers consider this finding dramatic because this metabolite is thought to be metabolically inert. They have also found that secretin is a mild but definite secretagogue for PTH. In

addition, they have found that amino-terminal antisera are particularly useful in preoperative localization of abnormally functioning parathyroid glands. These studies appear to have immediate relevance to the diagnosis and treatment of derangements of calcium and phosphorus metabolism. The other calcium-controlling hormone, calcitonin, has been purified from rats and the complete amino acid sequence of this hormone has been determined. This is an important step since rats are the experimental animals of choice in studies of this type.

For many years, a considerable number of patients have been afflicted with Paget's disease of the bone and there has been no effective therapeutic agent available to alter the course of this disease. At the present time, the hormone calcitonin and an additional substance known as ethane 1,hydroxy 1,1 diphosphonic acid, are being employed by researchers and they appear to be effective in treating this disease.

Gonads

A series of studies has been made to show the effects of alcohol on gonadal function. Male chronic alcoholics often suffer from features of hypogonadism and abnormal metabolism of sex steroids. Studies have shown that certain enzymes in the liver which normally control testosterone metabolism, have increased activity following chronic ethanol consumption in both rats and in human volunteers. It is felt that this increased enzymatic activity may be the cause of the altered androgenic activity in a chronic alcoholic. Additional studies indicate that not only is plasma testosterone metabolism increased following ethanol consumption, but also there may be an actual decrease in secretion of the hormone from the gonads. Studies have also shown that in some cases at least, chronic alcoholics seem to have a decrease in the amount of LH secreted from the pituitary gland. The conclusion reached from these studies is that the effect of ethanol on the metabolism of testosterone is a complex phenomenon involving both alterations in peripheral metabolism of the steroid hormone and central nervous and gonadal mediated events.

Metabolism Program

The Metabolism program area of the National Institute of Arthritis, Metabolism and Digestive Diseases supports, through the grant mechanism, projects concerned with developing new basic knowledge that will serve to increase the understanding of normal and disease processes. Unlike the categorical programs of the Institute, i.e., diabetes and arthritis, the metabolism program is not directed specifically to the understanding of any one particular disease process, but serves to foster knowledge development that impacts upon the numerous disease processes included within the programs of the NIAMDD as well as other programs throughout the NIH.

The Metabolism area emphasizes research related to increasing the understanding of the physical and chemical processes by which substances are maintained and produced by the living organism, as well as the processes by which energy is made available for use by the organism. Basic studies concerned with the mechanisms of enzyme action, biosynthesis of biological compounds, cellular oxidation, biological transport, control of enzyme actions, metabolism of substances, photobiology and physical biology -- especially as these impact upon an understanding of the diseases of man -- are included. A summary of the significant achievements of the program during FY 76 is as follows:

Biological Transport:

Calcium ions are involved in the regulation of many biological processes, including transmission of impulses along nerves, muscle contraction, photoreception, release of digestive juices from the pancreas and salivary glands, and cell division. When mechanisms for calcium control are disrupted or do not operate properly, the results are serious, and often lead to fatal, deforming, or debilitating diseases, such as the deposition of the calcified plaques characteristic of atherosclerosis, the appearance of seriously bowed legs in rickets, and the loss of supporting bone material that occurs in osteoporosis and which makes bone repair in older people so difficult.

In order to understand the functioning of calcium in both normal and abnormal tissues, the Metabolism program is supporting projects aimed at elucidiating the cellular mechanisms by which the movement of calcium to and from various body sites is controlled. Institute supported investigators have adapted the electron probe X-ray microanalyzer, an instrument primarily used in metallurgy and materials science, to these studies, so that they may follow the movement of calcium within and through individual cells. They have begun by studying two tissues through which a great deal of calcium normally moves. They have found that only certain cells pick up calcium at any one time while neighboring cells, which look identical, remain idle. They intend to identify what the stimulus is that causes these cells to become active. They have also found that each transporting cell contains much higher concentrations of calcium than had been thought and that the calcium moves through the cell in a compartment separate from the rest of the cell cytoplasm. They are attempting to

find out what keeps the calcium compartmentalized, and what mechanisms move the compartment from one surface of the cell to the other. Through this research investigators hope to be able to exert control over the flow of calcium through cells so that for example, abnormal loss of calcium from bone may be prevented and abnormal deposition of calcium in tissue may be alleviated or even reversed.

Enzymes:

Many types of abnormal functions in the body occur as a result of either the production of abnormal enzymes or in some cases, as a result of the absence of specific enzymes within the body. Such inherited diseases have been termed inborn errors of metabolism. It is now believed that an etiological component of Sudden Infant Death Syndrome (SIDS) may be correlated with abnormalities in the ability of certain children to synthesize glucose. NIAMDD supported investigators have compared the activity of certain gluconeogenic enzymes of liver from SIDS victims with that in liver from infants dying from other causes. It was found that phosphoenolpyruvate carboxykinase (PEPCK) activity was considerably lower in SIDS victims than in normal infants. In addition, a high percentage of the SIDS victims had an hepatic PEPCK that was defective in its response to certain metals. While these preliminary findings are stimulating considerable interest, the investigators still have no evidence that hypoglycemia is a cause of death in SIDS. For instance, some of the infants with low hepatic PEPCK activity had significant quantities of hepatic glycogen which suggest normal gluconeogenic processes. Low PEPCK activity could be a cause of fatal hypoglycemia, or it could merely reflect an adverse tissue response to the primary cause of death. Future support of this program by the NIAMDD and the NICHHD should provide some insight into this problem.

Biosynthesis of Proteins:

Proteins account for 17% of the total body weight and about 50% of all the organic material within the human body. No other class of organic molecule plays as many functional roles as does the protein. They are not only the basic structural unit of the body, but they also serve as enzymes that catalyze the various chemical reactions of the body, like the breakdown of foods and substances to yield energy.

The synthesis of these proteins in the cell is a highly complex process, involving as many as ten distinct steps. The organelle which serves as a template for the process is called the ribosome, and it is composed of many different parts. For example, ribosomes from some microorganisms are made up of some 55 different proteins and 3 different pieces of RNA. Many antibiotics exert their lethal effects by binding to the ribosome in such a manner as to inhibit protein synthesis. The current work of one Metabolism program supported investigation is directed toward identifying the binding sites of a number of antibiotics to ribosomes. The binding site of the protein synthesis inhibitor puromycin has been localized to one particular ribosomal protein (out of the total of 55). Combining results of this kind with what is known about antibiotic

inhibitory effects should allow for the construction of a detailed structure-function map of the ribosome. Information obtained for several different types of cell (e.g., bacterial, mammalian, malignant) may reveal differences in the mechanisms of protein synthesis which can be exploited in the design of chemotherapeutic agents which are much more toxic toward diseased tissue than toward normal tissue.

The pathways used by the cell to synthesize proteins constitute an important, complex, highly regulated part of the cellular mechanism used by the cell to express genetic information in chromosomes. Detailed information at the molecular and enzymological levels is essential in the understanding of these processes, and of the changes that take place in certain pathological conditions. It appears quite likely that alterations in the normal synthesis of proteins may occur in a number of diseases which may reflect altered controls of biosynthetic pathways. Defects in protein synthesis have been implicated in many genetically-based disorders, viral-infected or transformed cells, collagenous diseases, cystic fibrosis, tumor-related metabolic changes and senescence. Problems to the organism may be the result of changes in the structure of essential protein components, or in the regulation of their formation.

Current work in one Institute supported laboratory is concerned with the process of initiation in protein synthesis. This includes the manner in which the cellular machinery carries out the accurate recognition of the "initiation" signal, and the sequence of reactions that it undergoes between termination of one protein and the initiation of another. These reactions must be extremely precise and well-regulated if the cell is to avoid making random errors leading to the formation of inactive or otherwise faulty proteins. Several essential components concerned with initiation have already been identified, isolated and purified from normal tissues; their behavior in the appropriate recognition of the template messenger ribonucleic acid, and their role in the preservation of the protein synthesizing components that are not used up during translation, has been partly elucidated. The application of this new information to the study of various pathologies that appear to involve abnormal regulation is being developed.

Biosynthesis of Glycoproteins:

The attachment of sugar residues is one of the major modifications which a protein may undergo after synthesis of its peptide chains. A large number of proteins of diverse origin and biological function, including hormones, enzymes and many membranes are known to contain such tightly attached carbohydrate and are designated as glycoproteins. The process of linking sugars to protein involves a complicated enzymatic machinery which is susceptible to regulation by numerous normal and pathological factors. Disturbances in this sugar attachment process can lead to the production of abnormal molecules which can no longer carry out vital processes and therefore results in clinically detectable disease states. Glycoproteins have been implicated in numerous pathological conditions ranging from the abnormal behaviour of cancer cells to the capillary wall alterations of diabetes.

Institute supported investigations have contributed to a fuller understanding of glycoprotein synthesis in higher organisms by isolating and characterizing many of the enzymes and intermediates involved as well as their response to various regulatory factors such as hormones. Recently they have described a number of interesting molecules which could be involved in attaching sugars onto the surface of the cell.

These same investigators have been able to demonstrate that the membrane lining of the capillary wall is chemically altered in diabetes due to an overproduction of sugar-rich glycoproteins and this in turn can lead to its altered function which they believe may be responsible for blindness and kidney failure in the diabetic. The enzymes attaching sugars to the capillary membrane are overactive in diabetes but fortunately can be restored to normal by careful treatment of the disease with insulin.

Malignant cells may be distinguished from their normal counterparts on the basis of significant differences in the rate of synthesis, external surface exposure, and structures of glycoproteins that constitute the major structural components of cell membranes. A detailed understanding of these differences may provide a key to explaining many of the metastatic properties of cancer cells.

Institute supported investigators are studying these aspects of cell membrane biochemistry in malignant human lung cells, grown in laboratory cultures and, as a basis of comparison, are also studying these phenomena in the normal human lung cells from which the malignant cell culture was derived. Because human cell membranes contain a large number of individual glycoproteins, these investigators grow cell cultures under conditions which permit the cells to produce only one of its membrane glycoproteins. Thus, this approach allows them to focus their studies in great detail on the capacity of malignant cells to synthesize a single, major membrane constituent and avoid the confusion created if all of the membrane glycoproteins were being produced. With the aid of metabolic studies, detailed structural analyses, and the use of immunological approaches, the investigators hope to clearly define the unique features of glycoprotein production in malignant cells, and, ultimately, to be able to contribute to our understanding of the role that these tumor-specific macromolecules play in the invasiveness and accelerated growth properties of cancer cells. These studies with human cancer cells are complemented by a similar experimental approsch using myeloma cells that are cultivated as a malignant tumor in laboratory animals.

Photobiology:

Recent work in fundamental biological research and molecular biology has shown that the properties of living systems depend upon the shape and interactions of the molecules of which they are composed. In other words, it has been found that chemistry provides a basic explanation for many of the key processes of life, including heredity, specific recognition of invading foreign molecules, and communication between the cells of which animals are made.

One way of understanding how molecules interact is to study their responses to light beams and in part to analyze how they emit light after the light beam is shut off. This process, which is known as fluorescence, can be analyzed in very great detail using modern instruments. It allows one to obtain basic information on molecular collisions, molecular motions, and on the nature of the binding of one molecule to another.

Using this approach, Institute supported investigators have been able to analyze some of the fundamental properties of protein molecules including their response to heat and to various chemical manipulations. They have also analyzed the very important molecules of immunity called antibodies, that bind with foreign organisms and help to prevent disease. The interactions of foreign molecules with these antibodies can be traced using fluorescence measurements. In order to understand these interactions, these investigators determined for the first time in 1969 the entire molecular structure of a human antibody molecule.

Another immunological problem has also been fruitfully studied using this approach. This problem is to determine how the key cells of immunity, known as lymphocytes, grow, divide, and give rise to daughter cells that make more antibodies. These same investigators have used a protein from a plant that stimulates the division and growth of lymphocytes as a kind of molecular monkey-wrench. By analyzing the molecular architecture of this protein, called Concanavalin A, they have been able to dissect out the first steps of control of the growth of lymphocytes, the key cells of immunity. Fluorescence analysis played a key role in the structural part of this work.

Metabolic Control:

Of particular interest to the program is the area of metabolic control. Many pathological states are considered to be results of abnormal cellular metabolic control, e.g., cancer is characterized by uncontrolled cellular growth rate, diabetes is characterized by defects in the process by which glucose levels in the body are controlled. Recognizing the importance of these control processes, the Metabolism program is supporting numerous project areas aimed at understanding the cellular control mechanisms. One substance that has been implicated as playing a key role in cellular control mechanism is cyclic AMP.

Over the last decade it has become apparent that cyclic AMP, discovered as a consequence of its role in glycogen metabolism, plays an essential role in a vast number of bioregulatory phenomena such as cell growth and differentiation. The impact of this realization has been reflected in a redirection of investigative efforts to understand disease states as seemingly diverse as cancer, immunological deficiencies, endocrine abnormalities and diabetes mellitus. The Metabolism program is supporting several projects directed toward understanding how the actions of cyclic AMP are mediated. One group of investigators has purified the proximal receptor for the action of cyclic AMP, a cyclic AMP-dependent protein kinase from bovine cardiac muscle,

and have analyzed its molecular structure and its ability to phosphorylate cellular proteins thereby activating or inactivating cellular metabolic pathways. Recently they have focused their efforts on how its action is regulated by cyclic nucleotides. They have found that this protein kinase is composed of two dissimilar subunits: a cyclic AMP-binding protein containing two phosphorylated peptide chains, each of which binds a molecule of cyclic AMP, and two catalytically active subunits. When cellular cyclic AMP levels rise, cyclic AMP binds to the binding protein component of the protein kinase releasing active catalytic subunits from the native molecule. As cellular cyclic AMP levels fall, a phosphoprotein phosphatase dephosphorylates the free cyclic AMP-binding protein enabling it to recombine rapidly with the catalytic subunit to regenerate the inactive protein kinase. The protein kinase then phosphorylates itself to yield the native form of the cyclic nucleotide receptor protein. The principal feature of this complicated cycle is the interconversion of the phosphorylated and non-phosphorylated cyclic nucleotide-binding protein. Since the actions of cyclic AMP are a function of the activated form protein kinase:free catalytic subunit, any mechanism which alters the proportion of free catalytic subunit/inactive protein kinase will alter the effectiveness of cyclic AMP. Regulation of the state of phosphorylation of protein kinase has the potential for altering this ratio and ultimately explaining what might go awry in states of uncontrolled growth or ineffective hormone action. This research opens the way for pharmacological manipulation of these processes with the very real possibility of ultimately regulating the actions of the "master regulator," cyclic AMP.

A normally growing cell is said to become cancerous when its normal growth processes becomes uncontrolled. In other words, any cell in the body has the potential of becoming a cancer cell. A number of factors have been implicated as being involved in inducing cancer growth from normal tissue, however, underlying factors that transform a normal cell into a cancerous cell are still unknown.

DNA polymerase is a key enzyme in the growth and replication of cells in tissue. It is the enzyme that synthesizes DNA by polymerizing or building up this large molecule from small substrates. The enzyme accurately copies a DNA single helix in forming the double helix of DNA, making 100 - 1000 times fewer mistakes than can be predicted from the known double helical structure of DNA. Hence DNA polymerase enzymes are "too smart" -- they apparently possess an error preventing mechanism. These enzymes all require the addition of a metal ion such as Mg^{2+} or Mn^{2+} for activity. Binding studies of one metabolism supported project indicates that DNA polymerase functions by forming an enzyme-metal-substrate bridge complex in which the metal ion binds simultaneously to both the enzyme and substrate. This group has developed sophisticated methods (nuclear magnetic resonance) to measure distances, in solution, from Mn^{2+} to protons and phosphorus atoms of the substrates. From these distances they can build models showing how the enzyme-bound substrates are folded, and it is apparent that substrates are folded differently when they are bound to enzymes than when they are free in solution. On DNA polymerase the substrates are folded in such a way

that they will fit precisely into the double helix of DNA, the product of the enzyme reaction. The pre-folding of the substrates into the shape they will take in the product, may well be the enzyme's way of preventing mistakes. There is evidence that in cancer cells the DNA polymerase enzymes do not have this ability to prevent mistakes, i.e., the enzymes are "stupid." They may therefore be unable to fold the substrates properly. In principle, advantages could be taken of this difference between normal enzymes and those from malignant cells to design drugs to selectively inhibit the latter ones.

Split of the Metabolism Program: In the summer of 1975 the Metabolism Program was split and a new director appointed for Metabolism while its former director moved to the Metabolic Diseases Program. While the new program is much smaller it contains many projects in which the most sophisticated techniques are being used to dissect out the abnormal patterns of metabolism characteristic of the various diseases and spotlights many gaps in knowledge which need to be filled.

The current program includes the basic studies of lipid, purine, pyrimidine, urea, and polyamine metabolism as well as such diverse diseases as Cystic Fibrosis, Lesch-Nyhan Syndrome, gout, glycogen storage diseases, homocystinuria, adenosine deaminase deficiency, Wilson's Diseases, amyloidosis, and alpha-1-antitrypsin deficiency.

The Polyamine Paradigm: Last year's report (Metabolism Program) emphasized the role of homocysteine, the polyamines, and the aldehyde oxidation products of the polyamines as the neglected parameters of metabolic regulation. An ever increasing number of investigators have joined the"Polyamine Team" in establishing the Polyamine Paradigm of Metabolic Regulation. Basically this is a system of regulation by analogs of common key metabolites such as glucose, pyridoxalphosphate, formaldehyde, and multivalent cations such as calcium, manganese, or zinc.

Flux of metabolite along a pathway such as to protein, fat, or glycogen synthesis or to degradation of stored nutrients to amino acids, glucose, or fatty acid is controlled primarily by enzyme complexes whose activity is a function of the conformation of the entire complex. The conformation of the complex is in turn controlled by the interaction of multivalent metals, substrates, products (or inhibitors), and other effectors at the active or allosteric sites of the complexes. In general the activity of these various parameters is held within relatively narrow range.

Glutathione and polyamines are present in very high concentration in actively metabolizing cells and carry highly reactive groups such as the labile gamma glutamyl and sulfhydryl groups of glutathione and the trimethylene diamine groups of the polyamines which are reactive with aldehydes. Theoretical considerations suggest that a significant amount of any aminoaldehyde formed in the metabolism of the polyamines should be combined with an additional molecule of polyamine to form a highly basic dimer with four or more charged amino groups. As such this dimer should be more effective than spermine in cross linking DNA or in setting the conformation of various proteins.

Mechanism of Action of Insulin: Circumstantial evidence has implicated both the polyamines and glutathione in the action of insulin. The Polyamine Paradigm explains a role for both and predicts other interactions which are being found to exist. Insulin probably catalyzes a dithio exchange reaction between oxidized glutathione and membrane proteins. The gamma glutamyl group of glutathione, however, is very labile and its loss uncovers the very weak amino group of cystinyl glycine. Weak amino groups react readily with carbon dioxide to form carbamates or with aldehydes to form Schiff bases. As the

new aldehyde binding sites are introduced, polyamine aldehyde maintains thermodynamic equilibrium, binding to the weak amino group and freeing up the trimethylenediamine to which it was previously bound. The net effect in the membrane is to make the protein more basic (two relatively strong amino groups in exchange for one very weak amino group) while in the cytoplasm or nucleic acid complex such as the polysome the highly anabolic spermidine is made available with simultaneous loss of the repressor dimer.

It is well known that the diabetic polysome requires a much higher concentration of magnesium to synthesize protein at any rate approaching that of the normal polysome and that the maximum rate observed in vitro is only a small fraction of that known to occur in vivo. This situation is also true for the synthesis of glycogen which is also under control of insulin. Maximum rate of RNA polymerization in vitro is observed with approximately one mmolar magnesium and one mmolar spermidine. Apparently both polyamine and polyamine aldehyde are required within narrow limits for maximum synthetic rates and insulin stimulates the rates by making new binding sites available for the aldehyde.

Glycogen Synthesis: Glycogen synthesis is well recognized as under control of insulin as is the phosphorylation of glucose to Glucose-6-phosphate. A key role in the liver is played by the glucose-6-phosphate transferase which can transfer its phosphate to water (a phosphatase reaction) or can utilize pyrophosphate or carbamylphosphate to phosphorylate glucose. It has now been found that spermidine and copper (presumably permitting autoxidation of some spermidine to its aldehyde product) alter the conformation of this membrane enzyme to favor phosphorylation and to hinder hydrolysis, thus effectively setting a lower liver and thus blood glucose level since glucose is freely permeable across its membrane. The net result of insulin's action is to convert pyrophosphate, an inhibitor of glycogen synthase, to glucose-6-phosphate, a positive effector.

Ornithine Decarboxylase: This enzyme is considered as rate controlling for the synthesis of polyamines and shows extremely rapid changes in activity with half lives in the order of ten minutes. The enzyme is also very labile and difficult to purify. This has now been accomplished with the enzyme from the eukaryote slime mold where comparably short half lives are observed. Loss of activity in this case is reversible, the disappearance of the active enzyme with a Km for pyridoxalphosphate of 0.25 micromolar being accompanied by appearance of a new activity with a Km approximately one hundred times as great. Since negative feedbacks utilize an end product to inhibit one of the earliest committed steps in the pathway, much interest attends the study of the kinetics of this enzyme in the presence of polyamine aldehyde. It has also been observed that ornithine decarboxylase relieves the inhibition of TNA polymerase which is to be expected if the decarboxylase is binding a common inhibitor.

Cystic Fibrosis: The Polyamines and Cystic Fibrosis was the subject of a GAP (Guidance-Action-Projection) Conference held at the Fogarty International Center the end of January. The polyamines are expected to draw additional consideration in the international conference to be held the end of May in Jerusalem with the title, "Cystic Fibrosis, Projections into the Future". The formal presentations are already in print and conference time is to be

utilized in discussion with the reports of the session chairmen to be completed before leaving Israel and the completed book to appear by mid-July.

Homocystein Intolerance: Last year's report emphasized the role of elevated levels of homocysteine in body fluids to the pathogenesis of many degenerative diseases. Since homocysteine is a potent aldehyde binder any change in its concentration should be reflected in the polyamine interactions. The BHE rat shows many degenerative changes and a profoundly altered pattern of metabolism in the liver. Baboons infused with homocysteine show thickening and smooth muscle cell infiltration of the intima of the aorta which is reversible on return to normal diet. A major goal of the Metabolic Diseases Program will be to obtain recognition of the probable intolerance to homocysteine (and thus methionine) in degenerative disease.

Lesch-Nyhan Syndrome: This syndrome associates over production of purines, palsey, self-mutilization, and a characteristic psychosis with a deficiency of the enzyme which transfers the phosphoribosyl group of hypoxanthine to guanine. How this enzymatic deficiency brings about such profound changes is not understood but the rate controlling step in do novo purine synthesis is thought to be the formation of phosphoribosylpyrophosphate whose rate is controlled by the levels of inorganic phosphate and pyrophosphate. Despite elevated levels of circulating dopamine-betahydroxylase, characteristic of elevated circulating catecholamines, in the blood of Lesch-Nyhan children, they show little response to adrnergic stimulation in the way of increased blood pressure.

ARTHRITIS PROGRAM AREA

The major emphasis in the Arthritis Program has been on a mechanistic approach to the rheumatic diseases related to their diagnosis, possible prevention, effective treatment and cure. Not only has there been interest in a better understanding of the diseases themselves but an intensive effort is being sustained to determine the initiating event or events that trigger the processes that inevitably lead to the multiple forms of arthritis now known and the rheumatic diseases in general. The program continues to support efforts directed against rheumatoid arthritis, degenerative joint disease and systemic lupus erythematosus, marked by studies such as connective tissue activation in rheumatic diseases, the biological effects of lysosomal enzyme release, immune complexes in rheumatoid arthritis, rheumatoid arthritis and autoantibody formation in systemic lupus erythematosus and the direct investigation of rheumatoid arthritis itself. In addition, studies are in progress to examine collagen in osteoarthritic cartilage, human cartilage proteases in arthritis, proteoglycans in aging and osteoarthritic cartilage, the effect of joint motion on articular cartilage, chondrocyte-matrix interactions in arthritic disease, forms of experimental arthritis, the role of phospholipids in the mineralization of bone, the mechanics of normal, arthritic and prosthetic joints, and interests in the properties of synovial fluid. Finally, there are active interests in the significance of anti-nucleic acid antibodies in systemic lupus erythematosus, together with the mechanisms of tissue injury and nuclear antigens, the potential reversal of autoimmune tissue damage, virological studies of systemic lupus and the pathogenesis and therapy of the disease and its allied drug-induced state.

Many other supported programs and projects are concerned with substances or structures directly involved and related to these areas of major emphasis, and a better understanding and greater knowledge of the characteristics and functional properties of these structures and substances will inevitably lead to the better understanding of the diseases themselves and in some cases their associated musculoskeletal disorders. These are the programs concerned with immunology, including autoimmunity and hypersensitivity and disturbances of immune systems; skeletal muscle structure and function; the structure, function, production and biochemical studies of collagen, elastin, and connective tissue; and the ground substances such as the mucopolysaccharides and mucoproteins.

A singular event of great significance occurred during this fiscal year that was marked by the development and presentation to the Congress of the United States of the National Arthritis Plan which followed a twelve-month study and survey by the National Commission on Arthritis and Related Musculoskeletal Disease. The plan as it impacts here calls for a greater and more concerted effort in the pathogenesis, etiology, and treatment of rheumatoid arthritis and degenerative joint disease. Further, it calls for greater and specific initiatives in the arthritides of children, systemic lupus erythematosus, crystal induced arthritis, musculoskeletal diseases and joint structure and replacement. Finally, it suggests that basic biological research relevant to the rheumatic diseases be expanded and encompass infectious agents, developmental and cellular biology, immunology, inflammation

and the inflammatory processes, genetics and the structure and function of cartilage and connective tissue. The Arthritis Program, as a result, will have to accommodate for this expansion and develop initiatives in certain of the areas proposed. The Arthritis Plan calls for levels of training and manpower development consistent with its recommended goals and objectives and to be phased in at a definite rate. While the Arthritis Program continues to support training and manpower development under the National Research Service Act, it does not seem to be at a rate that will keep pace with the requirements of the Arthritis Plan or the rapidly diminishing support for the traditional training programs.

Chronic rheumatoid synovitis has been visualized as an exaggerated normal response to continuing injury based on the aggressive behavior of connective tissue cells. The possibility has been raised that connective tissue cells derived from rheumatoid synovial cells are a primary locus of the pathogenetic mechanism and/or etiologic factor responsible for rheumatoid inflammation. Connective tissue "activation" as a likely step has been delineated and a controlling substance identified as a low molecular weight basic protein containing vital sulfhydryl functions. Further study of this process might well provide insights into the basic mechanisms of the chronic inflammatory process, since the controlling substance can be shown: to induce normal synovial cells to perform biochemically in a manner typical to synovitis; to induce some of the "rheumatoid defects" in normal cells; and to be present in excess in rheumatoid cells.

Studies have been carried out concerned with the direct assessment of a fluid phase discovered at sites of calcification in small animals using renal micropuncture methodology with the concurrent development of reliable preparatory and analytical intramicro methods for studying the calcification process - and its inhibition - in samples of cartilage lymph aspirated from growth cartilage. The intent is to quantitate an array of biochemical parameters in micropuncture fluids as well as minute whole tissue samples eventually to be obtained by arthroscopy from patients with chondrocalcinosis and osteoarthritis as part of diagnostic workups. Based on recent findings, it is entirely plausible that there is an overlooked disorder of mineral ion metabolism in a substantial fraction of patients with degenerative joint disease.

The natural history of systemic lupus erythematosus is being studied in a genetically defined inbred dog colony to isolate and identify the transmissible agent associated with the canine form of this disease and to identify it as the causative agent. The intention is to apply the experimental systems used in the canine disease to human lupus and to clarify the relationships which exist between the lupus agent and oncogenic murine RNA viruses. Further, there is interest in the identification and characterization of viral antigenic determinants which may be common to the human, canine, and murine forms of systemic lupus. An immunofluorescent assay system and two radioimmunoassay systems specific for the SLE agent have been developed and attempts are being made to use these assay systems to screen human patient serum for antibodies to the lupus agent and as analytical tools to more clearly define the specificity of antibodies as they are found.

The anatomic distribution and morphologic character of immune complexes present near the surface of rheumatoid cartilage fragments available from human synovial membrane removed as a result of rehabilitative surgery was studied using a peroxidase-antibody reagent which appeared to be an excellent tool. The immune complexes are a unique feature of rheumatoid cartilage, not seen in cartilage from a variety of other conditions, and appear to be localized in the upper 100 micra of the cartilage surface adjacent to the joint space in the region generally devoid of most ground substance. They appeared amorphous and were often intimately associated with the surface of collagen fibers present in this cartilage layer. While no clearly identifiable morphology of antigen was seen, such as viral structures, antigen presumably exists in these complexes as amorphous material without regularly identifiable structure. In addition, a more systematic approach has been made to the problem of poor penetration of analytical reagents into intact pieces of rheumatoid synovial membrane. Previous attempts to study lymphocytes prepared from rheumatoid synovium have been hampered because of low cell recovery following isolation procedures with these tissues. A modified procedure used originally with liver cells and a procedure using a combination of chondroitinase and DNA-ase followed by collagenase digestion have provided encouraging results. A number of important studies of lymphocyte type and function should be possible if viable cells from rheumatoid synovium can be consistently produced in this manner. These studies provide an excellent opportunity to understand the mechanism of synovitis - the principal manifestation of rheumatoid arthritis. The dense infiltrate of lymphocytes in the rheumatoid synovium is compatible with a local immune response to the inciting antigen responsible for the disease. The immune complexes referred to, which have been localized to the superficial layer of rheumatoid cartilage, in all likelihood result from trapping of the antigen by locally produced antibody influenced by added rheumatoid factor. Further study could provide direct insight into the cause of rheumatoid arthritis.

DERMATOLOGY PROGRAM AREA

As noted in the previous year's annual report, the Dermatology Program of NIAMDD and the Allergy and Immunology Branch of the NIAID finalized plans for a jointly sponsored Immunodermatology Workshop. On October 22, 1975, 48 scientists met in Augusta, Michigan for a two and a half day program. Those attending were research dermatologists oriented in immunology or with interests having immunologic implications, and immunologists whose work has been identified as applicable to skin diseases. It has been recognized that with an increasing understanding of allergic and immunologic mechanisms of disease, continued investigations will help to resolve the many problems and disorders of the skin that remain to be defined. Accordingly, it was felt that needed interaction between the two groups invited could be a useful pursuit -- a workshop designed for the introduction and exchange of data and information and the generation of new ideas to be an important first step in developing a specialized interdisciplinary endeavor -- immunodermatology. The design of the program centered around five major areas of concern: (1) IgE-related cutaneous problems, (2) immune complexes in complement and cutaneous diseases, (3) delayed type hypersensitivity and skin disease, (4) cell mediated immunity and skin diseases, (5) diseases with multiple immunologic mechanisms and responses. The workshop was deemed to be highly successful. With the sorting out, examination and identification of problem areas of cutaneous disorders requiring further work and resultant stimulation of research initiatives through new immunologic approaches, longer range goals for the advancement of diagnosis and treatment of skin related diseases can be set. Publication of the proceedings in a forthcoming issue of the Journal of Investigative Dermatology will provide for prompt and widespread dissemination of this new and important information uniquely common to workers at the interface of immunology and dermatology.

More than six years ago, the NIAMDD identified psoriasis as a disease affecting millions of Americans and having a profound physical and/or psychological effect on many of these afflicted patients. It was felt that research in this disease and other hyperproliferative disorders of the skin required additional funding and encouragement. Over this period this Institute supported three national workshops and utilized the expertise of investigators from around the world to identify the areas in which fruitful research could be undertaken. Among the areas identified were those concerned with blood vessels in psoriatic plaques, control of proliferation in psoriatic epithelium, the pathways and dynamics of differentiation in the abnormal skin, the use of tissue culture techniques and the development of other models for the disease, application of immunogenetic information to the analysis of psoriasis, and studies of cyclic nucleotides, prostaglandins, and other mediators in the disease. It was also concluded that the cell membrane and its receptors should be studied and that unanswered questions concerning dermoepidermal interactions could and should be solved. The NIH-supported meetings were in part prophetic and in part stimulatory. In late 1974 the description of a new technique for the therapy of psoriasis was described by investigators at the Massachusetts General Hospital. Based on the pharmacologic principle of photochemotherapy, this technique, which involves the systemic administration of psoralen and the local administration of long-wave ultraviolet, is currently being evaluated in a nationwide

cooperative clinical trial. The NIAMDD is currently negotiating with representatives of the cooperative study for possible contract support of a long-term follow-up of patients treated with this new technique.

A direct result of NIAMDD-initiated activities was a contract awarded in FY 1975 for the study of previously unused topical chemotherapeutic agents in the therapy of psoriasis. The prime contractor is the University of Miami Department of Dermatology. This study, however, will also involve a nationwide cooperative approach.

Acne is a major disease entity in terms of patient-years of involvement, number of physician consultations and expenditure on medications. Current treatments are often ineffective, particularly in severe forms of the disease. Grantee supported efforts have been concentrating on the metabolism of sebaceous lipids because they are widely considered to be involved in both the initiation and maintenance of inflammatory acne. Lower levels of linoleic acid may be one of the factors related to the development of follicular hyperkeratinization in acne. The lower levels of this acid may also be related to increased inflammation due to increased prostaglandin synthesis. Institute-supported grantees are currently focusing more effort on the substantiation of the inverse correlation between linoleic acid concentrations in sebum and the occurrence of inflammatory acne. They are attempting to determine the source of linoleic acid by measuring the concentrations of this fatty acid in the wax ester, sterol ester, triglyceride and free fatty acid components from acne patients and normal subjects. They also hope to show whether the lower levels of linoleic acid in sebum from acne patients results from (a) lower dietary intake, (b) lower rates of biosynthesis, (c) the higher rates of sebum production in acne patients, (d) less efficient incorporation from circulating lipids by the sebaceous glands of acne patients, and (e) the effects of microbial action or some other mechanism.

Activities generated in FY 76 include plans to work closely with liaison members of American dermatology in order to develop projected needs for the training of research scientists within dermatology in order to increase their inadequate research manpower pool. A second immuno-dermatology workshop will be planned for the future and will focus its second meeting on more specific disease areas requiring attention. This will include atopic dermatitis and allergic contact dermatitis. A planning meeting will also be organized to determine the magnitude of a bizarre disease that results in striking loss of pigmentation of the skin, vitiligo. This disease affects all races but obviously exerts more profound effects on those patients with naturally darker pigmented skin.

ORTHOPEDICS PROGRAM AREA

Basic as well as clinical studies of bone in the normal and diseased state comprise the orthopedics program in NIAMDD. It includes projects on calcium metabolism relating to bone formation and repair, fractures and the healing process including electrical stimulation of healing, transplantation and preservation of skeletal tissue, surgical orthopedic implants and the biomechanics and bioengineering related to them, musculoskeletal dynamics and the physiology of normal and abnormal gait.

The total budget in orthopedics program for FY 76 is $7,846,000. There are currently 97 active research grants, including 1 program project, 10 training grants, 8 fellowships, and 6 RCDA's. This is approximately the same number of projects active last year at this time except for training grants, which are diminishing in number. Recently new training legislation was passed. Hopefully it will allow us to increase our training effort which has been waning steadily and needs expanding.

On March 5, 1976 a symposium was held on the Retrieval and Analysis of Orthopedic Implants sponsored by the National Bureau of Standards. NIH participated in this symposium which was designed to (1) assess the current status of implant retrieval, (2) determine the current state of knowledge of implant performance, (3) determine the additional information needed on implants and materials to improve implant performance, and (4) define areas where more fundamental information is needed. A final report of the symposium will be published.

In December 1975 a Workshop on the Status of Orthopedic Research and its Future Needs under the sponsorship of the American Academy of Orthopaedic Surgeons and the NIAMDD was convened in New Orleans. Approximately 70 leaders in orthopedic research gathered to discuss (1) where orthopedic research is today, (2) the areas where research is needed, (3) the manpower needed to carry on this effort, (4) funds necessary to adequately support orthopedic research, and (5) how NIAMDD can aid in all these endeavors.

Several areas of the orthopedics program encompassing both clinical and basic research have made significant advances during this fiscal year. They include:

Artificial Joints. Research and development of artificial joints is making steady advances. Now, artificial knees are being implanted by numerous orthopedic surgeons. A new prosthetic knuckle has been developed which enables deformed hands to grip and pinch. Additional experimentation is taking place on prosthetic ankles, shoulders, and elbows. New materials for these prostheses are constantly being developed, tested, and perfected. Materials currently used include metal alloys, graphite, ceramics and plastics and combinations of these. Recently an NIAMDD grantee evaluated a cobalt based alloy (31% N; 35% Co; 20% Cr and 10% Mo). This alloy possesses high tensile strength and good ductility, toughness and corrosion resistance. Implantation for up to 12 months produces a degree of local tissue response comparable to stainless steel. The characteristics of this new cobalt based alloy make it a promising material for permanent implants such as total hip prosthesis where there are long-standing cyclic stresses.

Mucopolysaccharidoses. An NIAMDD grantee has discovered that there is a delayed maturation in the pathway of crosslinking of the collagen fibrils in the bones of patients with osteogenesis imperfecta, a hereditary connective tissue disease. This delay may be due to either (1) a disturbance of stabilizing steps of the crosslinks or (2) genetic expression of different types of collagen in which the crosslinks are impaired. Further research should uncover more details concerning this disease of which little is known.

An NIAMDD grantee has discovered that in iliac crest biopsies from children with spondyloepiphyseal dysplasia congenita (SDC) contain increased amounts of chondroitin 6-sulfate and keratin-sulfate. This increase is similar to that observed in cartilage of patients with Morquio's disease but SDC patients did not excrete abnormal amounts of keratin sulfate in the urine. Hence SDC can be considered a mucopolysaccharidosis without mucopolysaccharidurla.

Bone Metabolism. A grantee of the orthopedics program has discovered that by giving intravenous cell suspensions prepared from spleen and bone marrow from normal littermates, mice with inherited osteopetrosis have permanently restored the capacity to resorb bone and calcify cartilage. This reversal of osteopetrosis through bone marrow transplantation is an exciting discovery which provides a new rational for treatment of Ablers-Schonberg marble disease in man.

Another grantee has developed a technique using dual photon absorptometry to precisely and accurately measure the bone mineral content in the lower spine and total body. This fast, improved method can be very useful in evaluating osteoporosis and other skeletal abnormalities.

Fractures and Fracture Healing. A grantee of the orthopedics program has perfected a method utilizing a silver anode to successfully treat dermal ulcers, infected bone non-unions and other long bone infections. This method, which appears to be free of the side effects which usually accompany systemic or local antibiotic therapy, can be very helpful in clearing up these chronic troublesome conditions.

Another grantee of the orthopedics program has perfected an ultrasonic device which provides information about the early healing and callus formation of bone fractures in dogs. This device should aid in evaluating the healing process and the effectiveness of the treatment of fractures, thus facilitating early management decisions and shortening total length of disability.

An NIAMDD grantee has made mechanical measurements of the stiffness of knees in rabbits after developing contracture in an immobilized hind limb. These animals who had estradiol injections during the nine weeks of immobilization had the stiffness in the immobilized leg reduced by approximately 50%. This discovery too could have definite clinical usefulness.

Until recently few studies have delved into the biochemistry of the healing of fractures. During the past year two separate studies have begun to throw light on this important process.

Bone collagen contains high levels of hydroxylysine during embryonic development. As maturation occurs, the level of hydroxylation is reduced to that normally associated with type I collagen of bone. An investigator has examined tissue identified as bone from cortical defects in adult chickens. Collagen extracted from these tissues is type I; however, the high level of hydroxylysine present indicates that osteoblasts in a repair situation in the adult "revert" and produce a high hydroxylysine-containing collagen similar to that produced in the embryo.

Histological studies suggest that fracture repair contains elements of both enchondral and membraneous bone formation which differ in amount relative to apposition and immobilization. An NIAMDD grantee has studied biochemically and metabolically the healing fracture callus. For the first 14 days the high hexosamine content is indicative of an early cartilage proteoglycans stage at the time of relative fracture instability. At 14 days the 30% mineralization, still in the presence of a high hexosamine content, is consistent with endochondral calcification. By 21 days the cartilage proteoglycan content markedly decreases together with an increase in fracture stability and further mineralization. Within the first six weeks of fracture healing, the callus accumulates both type I and II collagen. At 14 days 40% of the collagen accumulated is type II collagen, but at six weeks when the fracture is stable, approximately 20% of type II collagen remains. The presence in the fracture callus of cartilage type II collagen provides biochemical evidence to substantiate the concept of endochondral fracture healing. Further studies in this area are continuing. They could unravel the basic mechanisms of healing and add a whole new dimension to the study of the healing process.

OFFICE OF THE ASSOCIATE DIRECTOR
For
KIDNEY, UROLOGIC, AND BLOOD DISEASES
ASSOCIATE DIRECTOR'S REPORT

The Kidney, Urologic, and Blood Diseases (KUBD) Program has had a very active year which has focused on: evaluation of research in nephrology and urology; preliminary plans for evaluation of hematology research; increasing efforts to improve communication and cooperation with other Institutes with whom NIAMDD has common or overlapping research interests; support of workshops and conferences on topics where innovative research approaches are needed; and preliminary efforts to obtain reasonable epidemiological and statistical data about KUBD. These foci complement and supplement the research reported in each program.

The Evaluation Study of Research Needs in Nephrology and Urology (supported by a contract with the University of North Carolina; Principal Investigators: Dr. Carl W. Gottschalk and Dr. William E. Lassiter) is in its final stages. The eleven specialty committees' reports have been submitted to the chairmen and coordinating committee. The final document, including recommendations and the summary, is being completed. Overall research patterns, suggestions for new research initiatives and directives, and recommendations have been considered.

The commitment of NIAMDD to fundamental and clinical research in hematology continues. The appointment of David G. Badman, Ph.D., as Program Director for hematology, in November 1975, has given strong leadership in this area. Preliminary consideration has been given to evaluation of hematology research, but decisions will await the presentation of Dr. Yale Nemerson's survey of NIH support of blood research to the NHLI and the first meeting of the Blood Coordinating Committee.

Initial and/or continuing efforts for communication and cooperation with NHLI (Hypertension and Kidney Program, Division of Blood Diseases and Resources); NIAID; NCI; and FIC have been made. Further efforts will be made to enlarge this area of communication so that collaboration can be undertaken where possible and overlap minimized.

Because of the paucity of data about incidence and prevalence of kidney and urinary tract disease (KUTD), two projects have been initiated. The first is a contract (A Study of Population Data) with Kaiser Foundation of California (Principal Investigator, Gary Friedman, M.D.) to obtain data in their special population. A study of KUTD in the military services is in process and will utilize tapes of data from the three services--in collaboration with the Walter Reed Army Institute of Research, Department of Epidemiology.

In an effort to encourage investigator-initiated research into problems of urolithiasis which has considerable morbidity and which needs much research into its basic mechanisms, support has been given to several symposia on urolithiasis. These include the International Symposium on Urolithiasis Research, March 1976, held in Switzerland, and an International Symposium on Bladder Stone Disease, April 1976, Bethesda, Maryland. In cooperation with the Fogarty International Center a conference, Prevention of Kidney and

Urinary Tract Disease (PKUTD), was held at NIH in May 1976 and will be published as a monograph. Because means for prevention and/or arrest of PKUTD are few, this PKUTD conference emphasized research needed in ten areas of these fields. These are areas in which investigation is needed in order to find means to prevent or to arrest diseases which have high human or economic costs, significant morbidity and/or the possibility of progress with current technology or potential new developments. Support is planned for future small conferences on renal growth, Vitamin D metabolism, iron metabolism, and basement membranes.

In summary, the KUBD program is implementing efforts to encourage investigator-initiated research into basic mechanisms of disease through grant support and to identify areas requiring new research efforts. Initial approaches to identification of diseases requiring understanding through basic research include collection of data on human and economic costs and on incidence and prevalence of disease.

Kidney Disease and Urology Program

The Kidney Disease and Urology Program, now in its thirteenth year, has over 230 active research grants whose awards totaled $15.45 million during fiscal year 1976. More than 75 per cent of the research grants funded by this program support basic investigations relating to kidney and urinary tract function and their relation to disease. The Program's responsibilities continue to encompass the investigation of etiology, pathogenesis, diagnosis, and treatment of specific kidney and urinary tract diseases, within the relevant areas throughout all periods of life in which they may occur. The total program of research support includes studies of a very fundamental nature as they are associated with the more specific objectives, including: lupus nephritis, auto-immune processes of the kidney and urinary system; basic and clinical studies of the kidney and urinary tract in disease states such as nephritis, nephrosis, benign prostatic hyperplasia, polycystic disease, diabetic nephropathy, urolithiasis, glomerulonephritis, and glomerulonephropathies; pyelonephritis and other nephritides; renal regulatory mechanisms including the renin-angiotensin-aldosterone system; basic research and clinical studies related to dialysis and the uremic or anephric patient or experimental animal; basic and applied studies on renal transplantation including techniques of organ preservation and storage; renal function, including metabolic and transport mechanisms and their disorders. There are overlapping areas of interest with other institutes within NIH in their mutual interests in end-stage renal disease, renal function in transplantation, pyelonephritis, glomerulonephritis; electrolyte balance, vascular components of kidney structure and function; the paraplegic bladder and so forth. Also, there is overlap with other programs within the Institute relating to studies on Systemic Lupus Erythematosus, diabetic nephropathy; chronic renal failure, dialysis; anemia of uremia, and calcium and phosphorus metabolism.

Basement Membrane. The basement membrane is one of the most complex and poorly understood entities in the human organ system. In the kidney it is the only continuous membrane in the glomerulus and it is believed by many to be the renal filter. The results of five years of study at the University of Colorado Medical Center suggest that observations made in their laboratory could lead to new theories and a better understanding of the composition and function of the basement membrane. According to this group, their findings suggest that the biosynthesis of the basement membrane does not conform to the expected route for glycoproteins. Also, they have discovered and partially characterized an enzyme that degrades the basement membrane *in vivo*. In their judgement, these observations have led to a new concept of basement membrane. For instance, it is now known to be composed of a tropocollagen core to which is bonded an antigenic glycoprotein; arrangement of the molecules are as a mesh with a definable pore style. These investigators hypothesize that these pores are probably filled with soluble basement membrane, serum protein and/or ground substance, which serve as a mechanical filter by making use of the proe contents to serve as a chemical barrier or operate on the basis of ion exchange or molecular sieving.

Transplantation. Late rejection of renal grafts continues to be the most important problem in clinical transplantation and unraveling the mechanism of this rejection is of primary importance for the success of renal grafts. Although the major thrust of an investigator at D. C. Children's Hospital focuses on the application of chalones to immunosuppression, it was by serendipity that he discovered a bacterium that inhibits lymphocyte transformation. He reports that he has produced a bacterial-free supernatant extract that inhibits or paralyzes lymphocyte transformation in vitro and skin allograft rejection in vivo. The course of the inhibitor is a psychrophilic strain of Pseudomonas putida, which is destroyed by trypsin or chymotrypsin. It is not cytotoxic in vitro against a wide variety of human cells nor is it markedly toxic, even in large doses in mice. The crude extract from this nonpathogenic bacterium at a concentration of 10 micrograms per milliliter will give over 90 per cent inhibition of DNA synthesis in human and murine lymphocytes in two way mixed lymphocyte cultures. Finally, it was found that this extract does not deplete the medium of essential nutrients but, rather, seems to interact directly with the surface of lymphocytes, that is, inhibit capping. Once the investigator establishes the Minimal Effective dose, the LD-50 and the duration of the inhibitory effects in the circulation after injection, he will attempt to determine the effective dose to prolong renal allograft survival in rats and ultimately in dogs. Employment of the inhibitor as an agent for immunologic suppression bring a new dimension to the control of transplant rejection. For instance, this investigator suggests that this macromolecule might sharply reduce the toxic side effects inherent to the pharmacologic agents now routinely used for this purpose. Furthermore, its ability to inhibit DNA synthesis without apparent cytotoxicity should prevent the lymphoid cholonal proliferation requisite for transplantation rejection without simultaneous destruction of other lines of pre-existing immunity, such as the gratuitous destruction of macrophages, granulocytes or the activation of complement.

Kidney Stones. Kidney stones are a common disorder affecting at least one million persons per year in the United States. However, the exact cause and management of this malady have not been clearly understood. Recent studies involving approximately 200 patients at the University of Texas Health Science Center at Dallas have provided additional information on the most common form of stones -- those containing calcium. This information includes a better understanding of the causes of stone formation, a development of reliable techniques for the diagnosis of the different types of stones, and the formulation of various treatment programs which are directed at correcting the underlying causes. High urinary calcium (hypercalciuria) was found in 86 percent of the patients evaluated. In addition, a reliable method which could be adapted for outpatient use in the office of a practicing physician was developed to categorize different causes of high urinary calcium. The study revealed that 20 percent of the patients with calcium stones had resorptive hypercalciuria; high urinary calcium resulting from excessive destruction of bone by an overactive parathyroid gland (usually from a benign tumor). Fifty percent of the patients had absorptive hypercalciuria; absorbing excessive amounts of calcium from the diet. Ten percent of patients suffered from renal hypercalciuria, which was caused by an inability of the kidneys to adequately conserve calcium. The remaining patients had normal urinary calcium but some of them

had high urinary uric acid. Concurrent laboratory studies by this investigator have shown that high urinary calcium or uric acid may have caused calcium stone formation. Accordingly, various treatment programs were developed to lower urinary calcium or uric acid. He found that resorptive hypercalciuria is best treated by surgical removal of the parathyroid tumor, whereas the optimal treatment of absorptive hypercalciuria was found to be the oral administration of cellulose phosphate which specifically binds calcium in the intestinal tract. Also, it is his opinion that renal hypercalciuria is best treated with thiazide diuretics, purported to correct "calcium leak" in the kidneys. Allopurinol, which inhibits uric acid synthesis, is suggested as the best means to treat patients with high urinary uric acid. With all these forms of treatment, patients were found to form fewer or no stones as their urinary calcium or uric acid decreased.

In summary, these studies have allowed classification of kidney stone diseases, development of an easy and reproducible method of diagnosis, and formulation of optimal therapy for the various causes of stones. Thus, it is the contention of this investigator that this very common financial, social and medical problem can now be controlled by medical means and in turn has improved the health of kidney stone patients.

Hypothyroid Effect on the Kidney. An investigator at Columbia College of Physicians and Surgeons has shown that the growth of kidney tubules ceases in the rat at the time the thyroid gland is ablated, whereas the glomeruli continue to grow but at the retarded rate characteristic of the body as a whole. The most important potential significance of this work is that it may lead to an improved understanding of the factors determining renal growth and differentiation, which may be particularly helpful in attacking the problems of renal disease in infancy and childhood. An unexpected additional finding that plasma renin-substrate is greatly reduced in the hypothyroid rat calls attention to hepatorenal interactions that may be instrumental in the development of the kidney and in the pathogenesis of certain renal dysfunctions. Since it is now evident that hypothyroidism in man is also associated with a tendency to sodium loss, these studies of the rat should be helpful in elucidating the disease in man.

Metabolic Studies on Isolated Preserved Kidneys. Studies underway at the University of Oklahoma indicate that the operative procedures and warm and cold ischemia times do not seriously affect the biochemical processes which were measured and used as criteria to assess the metabolic viability of the preserved kidney. Of the biochemical pathways studied by this group, gluconeogenesis of the kidney cortex appeared to be the most informative measurement. For instance, glucose production from various substrates was decreased to a similar degree in hypothermic non-perfused or saline perfused kidneys by 24 hours. Measurements of the intermediary metabolites in the glycolytic pathway and of malate and ATP suggest that the decrease in glucose production from substrates which enter the metabolic pathway either in the glycolytic stream or at the level of the citric acid level appear to require energy and integration of highly complex pathways involving multiple enzymatic steps

between the entry point and the production of glucose. This process therefore reflects an important integrative functional activity of kidney cortex cells.

Training. Although the old T01 program is in the process of being phased out, support for thirteen T01 training programs were continued in FY 1976 and eleven institutional National Research Service Awards (T32's) were begun, which in the aggregate provides stipend support for 59 postdoctoral trainees. In addition, twenty individual fellowships were active during this period and three new Research Career Development Awards were awarded, increasing the number to nine.

Conference on the Prevention of Kidney and Urinary Tract Disease. The National Institute of Arthritis, Metabolism and Digestive Diseases and the Fogarty International Center jointly sponsored a Conference-Monograph on the Prevention of Kidney and Urinary Tract Disease at the National Institutes of Health on May 24-25, 1976. The Conference attracted 120 individuals to discuss many varied topics of the kidney and urinary tract, including: genetic disorders, glomerular diseases, hypertension, acute renal failure, obstructive uropathy, urolithiasis, urinary tract infection, interstitial nephritis, pregnancy and renal disease and evaluation of screening and health maintenance. The proceedings are to be published as one of the Forgary International Center Series on Preventive Medicine.

Benign Prostatic Hyperplasia (BPH). A Workshop on Benign Prostatic Hyperplasia, sponsored by this Institute in February 1975, provided an impetus for a number of investigators to submit applications seeking support to study this disease. A large program project grant was recently awarded to a multidisciplinary group at Johns Hopkins University which will support four years of investigations dealing with the development of an animal model, evaluation of assay systems, the prevention of spontaneous and induced BPH and an evaluation of the most critical etiologic factors.

A monograph covering the proceedings of the BPH Workshop is to be in print by August 1976 and will be distributed to 4000 individuals interested in and working on this important medical problem. An additional 1000 copies will be distributed to medical libraries and urological residency programs throughout the United States, Canada and Europe.

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS
AND SCIENTIFIC COMMUNICATION

SCIENTIFIC COMMUNICATIONS SECTION

Table of Contents

The Scientific Communications Section has responsibility for general programming and planning of communication activities concerned with various scientific areas charged to NIAMDD. The present activities can be generally grouped 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. At present, there are four publications with hierarchically arranged listings of literature citations and one publication which includes abstracts. GASTROENTEROLOGY ABSTRACTS AND CITATIONS, now in its ninth volume, is intended to provide selected abstracts and literature citations in all aspects of gastroenterology, biochemical or clinical. Four of the publications, KIDNEY DISEASE AND NEPHROLOGY INDEX, incorporating the ARTIFICIAL KIDNEY BIBLIOGRAPHY, (first volume), ENDOCRINOLOGY INDEX (ninth volume), DIABETES LITERATURE INDEX (eleventh volume), and INDEX OF DERMATOLOGY (eighth volume) 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).

To produce structured, hierarchically-arranged indexes which function as in-depth current-awareness tools for easy review of particular subjects, we have cooperated with the National Library of Medicine, and in one case also with the University of Minnesota and the University of Rochester.

The standardized terminology used in MEDLARS makes these publications invaluable for retrospective literature searching and to those who are interested in these highly specific areas.

Outstanding scientists working in the field covered by the publication have been assembled for each publication as editorial advisors to ensure the continuation of the high quality of these publications.

Expansion of the quarterly ARTIFICIAL KIDNEY BIBLIOGRAPHY to include all kidney disease, nephrology, and urology was accomplished with publication of the new bimonthly KIDNEY DISEASE AND NEPHROLOGY INDEX. With Volume I (1975) the issues were combined into two large issues. However, with Volume II (1976) the publication will appear with bimonthly issues.

Conferences and workshops sponsored by NIAMDD are planned and organized by the Scientific Communications Section. In addition to the organization of the conference with the conference chairman, (which usually includes physical arrangement as well as program planning), the publication of the proceedings also falls under the auspices of the Scientific Communications Section.

This year ten conferences and workshops, sponsored either completely or jointly, were held. These included the Ninth Annual Contractors' Conference of the Artificial Kidney-Chronic Uremia Program, the Study for Evaluation of Testing for Cystic Fibrosis, the Conference on Rheumatic Diseases of Childhood, the Second Annual Conference of the American Association for Clinical Histocompatibility Testing, the Conference on Ileojejunostomy for Obesity, Prevention of Kidney and Urinary Tract Disease, the Conference on Immune Mechanisms in Cutaneous Disorders, and the Symposium on the Development of Iron Chelators for Clinical Use. Workshops included the Workshop on Manpower and Research Funding in Dermatology and the Workshop on Anemia of Chronic Renal Disease.

The National Cystic Fibrosis Research Foundation has compiled a comprehensive bibliography with indexes covering all the known literature on various aspects of cystic fibrosis that will be available this fall.

The DIET GUIDE FOR PATIENTS ON CHRONIC DIALYSIS was revised and updated, reprinted twice, and will be offered for sale through the Government Printing Office this summer.

Proceedings of the Third International Conference on Bone Mineral Measurement, the third in a series on the subject of bone mineral measurement sponsored by the University of Wisconsin Bone Mineral Measurement Laboratory, will be published this summer.

The Proceedings of the 1972 International Bladder Stone Conference held in Bangkok, Thailand will be published through the Government Printing Office and available in the fall.

National Library of Medicine's on-line MEDLARS (Medical Literature Analysis and Retrieval System) is available to NIAMDD staff through NIAMDD's call number. The Scientific Communications Section is responsible for coordinating this use and is a reference source for the NIAMDD staff.

Project Title:	DIABETES LITERATURE INDEX
Contract Number:	PH43-67-663
Amount:	$102,221
Contractor:	University of Minnesota
Project Officer:	Mrs. Billie B. Mackey
Man Years:	Total: .52 Professional: .32 Other: .20

Project Description:

A monthly current-awareness publication, the DIABETES LITERATURE INDEX, is now in its eleventh volume. This bibliography was brought about through the cooperative efforts of the Institute, the National Library of Medicine, and the American Diabetes Association, and three universities, (University of Minnesota, University of Rochester, and Case Western Reserve University), and many individual scientists.

MEDLARS magnetic tapes containing all of the current biomedical literature citations used for the preparation of NLM's INDEX MEDICUS, are used to retrieve all diabetes-related bibliographic citations monthly. Until 1971 the DIABETES LITERATURE INDEX consisting of a keyword in title index and an author index resulted from a computer printout. From 1971 to date, the magnetic tape version of these indexes is prepared at the University of Minnesota and is used to generate copy for offset printing by the Government Printing Office on Linotron. This monthly publication consists of three formats, a hierarchical subject index of core diabetes literature, an abbreviated keyword in title index of all diabetes literature, and an author index including index terms with the citations (for the first author). Grouping the citations under a preconceived hierarchical structure, with related subjects together, makes it easier for users to scan their particular interests in one section.

DIABETES LITERATURE INDEX is published monthly by the Scientific Communications Section, NIAMDD, and is available to National Institutes of Health grantees or contractors with interests in diabetes, government agencies with responsibilities in this area, and medical school libraries. It is for sale by the Superintendent of Documents, Government Printing Office for a fee. The free mailing list was circularized to insure currency.

Project Title:	ENDOCRINOLOGY INDEX
Professional Personnel:	Mrs. Billie B. Mackey
Man Years:	Total: .7 Professional: .4 Others: .3

Project Description:

A bimonthly recurring bibliography, the ENDOCRINOLOGY INDEX, is now in its ninth volume. Each issue contains approximately 4,000 citations of the current world literature in endocrinology. Citations are grouped in eight categories (pituitary, thyroid, etc.), and each category has a preconceived hierarchical organization of subject headings. Index terms, assigned to the original article by the indexer, appear in the author section, and author and subject indices appear in each issue. This bibliography is produced, with the aid of the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS), by the Scientific Communications Section, NIAMDD.

Citations from other sources are selectively added, including citations of books of potential interest to endocrinologists which have been chosen from National Library of Medicine's CURRENT CATALOG as well as citations of books and articles of potential interest to neuroendocrinologists which have been chosen from the five publications of the Brain Information Service. Work with the Medical Subject Headings Section of the National Library of Medicine for inclusion of endocrine-related vocabulary in the computerized vocabulary is on-going.

ENDOCRINOLOGY INDEX is available to National Institutes of Health grantees or contractors with interests in the field of endocrinology, government agencies with responsibilities in the field, and medical school libraries. It is for sale by the Superintendent of Documents, Government Printing Office for a fee. The free mailing list was circularized to insure currency.

Project Title:	GASTROENTEROLOGY ABSTRACTS AND CITATIONS	
Contract Number:	NO1-AM-4-2204	
Amount:	$120,100	
Project Officer:	Mrs. Billie B. Mackey	
Other Professional Personnel:	Dr. Barry Kemler Mrs. Elsie C. Yuen	
Man Years:	Total:	.25
	Professional:	.15
	Other:	.10

Project Description:

Since January 1966, NIAMDD has been publishing GASTROENTEROLOGY ABSTRACTS AND CITATIONS, a monthly current-awareness journal for gastroenterologists. Prepared by contract, each issue contains approximately 350 abstracts and 750 citations of the significant world literature on gastroenterology. The abstracts and citations are listed under three main categories: Clinical Sciences, Diagnostic Procedures, and Gastrointestinal Diseases. Each issue contains a subject and author index which are cumulated annually.

The staff reviews and edits the abstracts and citations prepared by the contractor prior to publication.

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, and medical school 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. The mailing list was circularized to insure currency.

Project Title: INDEX OF DERMATOLOGY

Professional Personnel: Mrs. Elsie C. Yuen
Mrs. Billie B. Mackey

Man Years:

Total:	.5
Professional:	.25
Other:	.25

Project Description:

To support the NIAMDD Dermatology Program, and respond to an increased interest and demand for up-to-date clinical studies and research information in dermatology, NIAMDD has been publishing the INDEX OF DERMATOLOGY since April 1972.

The INDEX OF DERMATOLOGY is a monthly recurring bibliography produced by the Scientific Communications Section with the aid of MEDLARS, the Medical Literature Analysis and Retrieval System of the National Library of Medicine. About 1200 dermatology-relevant citations are computer-selected each month from the INDEX MEDICUS input for the corresponding month, and listed under general clinical, biology, and/or cell component sections, and under an author index. The hierarchical format used allows titles in a related subject area to be grouped, on the basis of their clinical or research orientation, under a specific subject heading.

The staff edits the camera copy of the bibliography prior to publication. Subject headings without retrieval are examined to assure that relevant citations have not been omitted. For 1976, close to 50 new subject headings, primarily dermatologic agents, have been added or formally recognized.

To keep the publication useful and timely, guidance is available from 14 outstanding dermatologists serving as Editorial Advisors. The Editorial Advisors usually meet with the NIAMDD staff yearly to discuss changes and improvements.

The INDEX OF DERMATOLOGY is available free to National Institutes of Health grantees or contractors who are involved in work related to dermatology, to other government agencies with responsibilities in the area, and to medical school libraries. In addition to the free distribution, it is for sale by the Superintendent of Documents, Government Printing Office, at a nominal fee. The mailing list was circularized to insure currency.

Project Title: KIDNEY DISEASE AND NEPHROLOGY INDEX

Professional Personnel: Mrs. Billie B. Mackey

Man Years:

Total:	.9
Professional:	.6
Other:	.3

Project Description:

Expansion of the quarterly ARTIFICIAL KIDNEY BIBLIOGRAPHY to include all kidney disease, nephrology, and urology was accomplished. The new KIDNEY DISEASE AND NEPHROLOGY INDEX, a bimonthly bibliography, covers kidney disease, nephrology, urology, artificial kidneys, and kidney transplantation. It is produced with the aid of the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS) by the Scientific Communications Section, NIAMDD. Each issue contains approximately 2200 citations with the citations arranged hierarchically to group similar subjects together. Subject and author indices are included. A listing of annual and final technical reports submitted to the Artificial Kidney-Chronic Uremia contract program are included. These reports are available through the National Technical Information Service for a fee.

The KIDNEY DISEASE AND NEPHROLOGY INDEX is available to National Institutes of Health grantees or contractors with interests in nephrology, urology, or artificial kidneys, government agencies with responsibilities in the area, and to medical school libraries. It is for sale by the Superintendent of Documents, Government Printing Office for a fee. The free mailing list was circularized to insure currency.

Project Title:	ARTIFICIAL KIDNEY-CHRONIC UREMIA PROGRAM EIGHTH ANNUAL CONTRACTORS' CONFERENCE PROCEEDINGS
Professional Personnel:	Dr. Robert J. Wineman Dr. Fernando Villarroel Mrs. Billie B. Mackey
Man Years:	Total: .4 Professional: .2 Others: .2

Project Description:

The Proceedings of the Eighth Annual Contractors' Conference held in Bethesda, Maryland, January 13-15, 1975 was published. About 175 participants who were contractors and their staff, consultants to the Artificial Kidney - Chronic Uremia Program, and Institute staff had attended the conference. The sessions were devoted to reports of existing contract-funded research and development projects with presentations grouped in three sessions: 1) Biochemistry and Pathophysiology; 2) Biocompatibility, Membranes and Blood Access; and 3) Therapy, Evaluation, and Device Development. The conferees communicated research results in the main areas of the Artificial Kidney - Chronic Uremia Program.

Project Title:	Artificial Kidney-Chronic Uremia Program Ninth Annual Contractors' Conference and Proceedings
Professional Personnel:	Dr. Robert J. Wineman Dr. Fernando Villarroel Mrs. Billie B. Mackey
Man Years:	Total: .4 Professional: .2 Other: .2

Project Description:

The Ninth Annual Contractors' Conference was held January 12-14, 1976. This year all sessions of the Conference were open to interested individuals with approximately 250 attending. About 175 participants who were contractors and their staff, consultants to the Artificial Kidney-Chronic Uremia Program, and Institute staff attended. The sessions consisted of reports of existing contract-funded research and development projects. The presentations were grouped in two sessions: (1) biochemistry and pathophysiology and (2) biocompatibility, membranes, and device development.

The conferees communicated research results in the main areas of the Artificial Kidney-Chronic Uremia Program and a considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of the 70 currently active research and development projects, the Program's consultants and its staff. This and future conferences will assist in attaining the Artificial Kidney Program's goals--optimum artificial kidney development, improved clinical methodology, and better patient rehabilitation. The Conference was held in Bethesda, Maryland.

The proceedings of the Conference will be published in the fall 1976.

Project Title: Artificial Kidney-Chronic Uremia Program
Workshop on Anemia of Chronic Renal Disease

Professional Personnel: Dr. Robert Wineman
Dr. Barry Kemler
Mrs. Billie B. Mackey

Project Description:

Anemia continues to be a frequent and severe complication of chronic renal failure. In order to investigate the etiology and therapy of anemia, the Artificial Kidney-Chronic Uremia Program held a workshop to determine the current state of knowledge regarding this problem and to facilitate the best approach to future investigation through the contract process. Approximately fifteen consultants and staff attended the workshop. The anemia of chronic renal failure is largely the result of both decreased red blood cell production and increased red cell destruction. In directing its research efforts, the Artificial Kidney-Chronic Uremia Program is interested in those approaches most likely to correct both of these conditions. It is through workshops such as this one that various routes of investigation may be uncovered and the most feasible route for research support determined. The workshop was held in Bethesda, Maryland, November 12-13, 1975.

Project Title: Conference on Ileojejunostomy for Obesity

Professional Personnel: Dr. Harold Roth
Mrs. Billie B. Mackey

Project Description:

Ileojejunostomy, prescribed as surgical treatment for malignant obesity, though effective, is nevertheless a controversial approach still in the trial stage. Adverse reactions such as liver damage have been demonstrated.

At the conference held at Foundation Headquarters in Santa Ynez from January 31 to February 3, 1976, twenty one specialists with relevant expertise from across the United States, England, and Denmark met to examine the effectiveness and risks of the procedure. They identified some factors that may be helpful in patient selection, discussed problems of managing such patients, and formulated recommendations.

Project Title: Conference on Rheumatic Diseases of Childhood

Professional Personnel: Dr. G. Donald Whedon
Mrs. Billie B. Mackey

Project Description:

There are probably about 400,000 children in the U.S. today afflicted with various rheumatic disease syndromes; 250,000 of these children have juvenile rheumatoid arthritis which can be a chronic and potentially crippling disease. Rheumatic diseases in childhood are usually amenable to therapy and the prognosis of affected patients is often improved with early diagnosis and appropriate therapy. The purpose of this conference was to define the knowledge now existing and to define the status of pediatric rheumatology in order to determine future directions. Approximately forty participants active in the field of children's rheumatology or having special knowledge about rheumatic disease in children attended. The proceedings will be published as a supplement to the journal, ARTHRITIS AND RHEUMATISM. The conference was held in Park City, Utah on March 21-25, 1976.

Project Title:	CYSTIC FIBROSIS BIBLIOGRAPHY
Contract Number:	NIH - NIAMDD - 72-2214
Amount:	$8,965
Professional Personnel:	Mrs. Billie B. Mackey Mrs. Elsie C. Yuen
Man Years:	Total: .15 Professional: .10 Other: .05

Project Description:

Cystic fibrosis is considered to be one of the most serious childhood diseases, and is now diagnosed with increasing frequency throughout the world at a rate of nearly 1000 new cases each year. As a cause of death in children, it claims far more victims than rheumatic fever, diabetes, and poliomyelitis which are better known. At present, cystic fibrosis research is directed toward clarifying the clinical manifestations of the disease and uncovering the basic defect responsible, as well as finding the best approach to treating the disease.

NIAMDD has supported publication in the past of two bibliographies prepared by the Cystic Fibrosis Foundation. The first, a 15-page pamphlet published in 1959, and the second, an 85-page booklet published in 1966, have provided scientists with a valuable research tool to better understand, treat, and control cystic fibrosis.

The present publication, CYSTIC FIBROSIS: A COMPREHENSIVE BIBLIOGRAPHY OF THE MEDICAL LITERATURE, 1813-1972 was designed to include all the relevant scientific information in the world literature on cystic fibrosis up to the present. It contains a citation list of approximately 5200 titles in the English and non-English languages, and topic and author indexes. The indexes are designed to help the user locate wanted citations with greater efficiency.

The bibliography will be available in the fall.

Project Title:	DIET GUIDE FOR PATIENTS ON CHRONIC DIALYSIS
Personnel:	Dr. Benjamin T. Burton Mrs. Billie B. Mackey Mrs. Elsie C. Yuen
Man Years:	Total: .10 Professional: .05 Other: .05

Project Description:

The amounts of sodium, potassium, and fluid in the blood of the artificial kidney patient on chronic dialysis must be regulated carefully to keep them from building up into toxic proportions. This is done by placing the patient on a special diet.

The DIET GUIDE FOR PATIENTS ON CHRONIC DIALYSIS was designed by a dialysis dietitian to encourage patients to adhere to their prescribed diets. This year it was updated and revised.

Because of increasing demand, this popular guide has undergone two reprintings. In the past, NIAMDD has supplied copies free of charge to physicians and dialysis centers. However, due to heavy demand, future copies will be available for sale by the Superintendent of Documents, Government Printing Office.

Project Title: Immune Mechanisms in Cutaneous Disorders

Professional Personnel: Dr. Laurence Miller
Mrs. Billie B. Mackey

Project Description:

The purpose of this workshop was to exchange research data among immunologists and dermatologists, to determine the status of current knowledge, to stimulate needed research into relevant areas of immunology and dermatology, and to translate these contributions to improve the health of those individuals with allergic and immunologically related dermatologic diseases. It is known that cutaneous involvement occurs only because the skin serves as a specific target organ in a systemic immunologic disorder or furnishes the antigenic stimulus or shares in the responsible immunologic process or allergic mechanism. Prime topics covered included psoriasis, atopic dermatitis, cutaneous vasculitis, immune complex diseases, bullous diseases, and cellular immunology. The proceedings are to be published in the JOURNAL OF INVESTIGATIVE DERMATOLOGY in the fall 1976. The conference was held at Brook Lodge, Kalamazoo, Michigan, October 22-24, 1975.

Project Title: Prevention of Kidney and Urinary Tract Disease

Professional Personnel: Dr. Nancy Cummings
Dr. James Scherbenske
Mrs. Billie B. Mackey

Project Description:

This conference grew out of a series of monographs sponsored by the Fogarty International Center on prevention of disease in categorical areas, specifically, the Prevention of Kidney and Urinary Tract Disease. NIAMDD sponsored the conference at which the authors presented their papers to a group of nephrologists, urologists, epidemiologists, and statisticians for discussion.

The areas covered which will appear as chapters in the monograph include genetic renal disorders, obstructive uropathy, glomerular diseases, urinary tract infections, interstitial nephritis, urolithiasis, acute renal failure, hypertension and renal disease, pregnancy and renal disease and evaluation of screening and health maintenance. The monograph will appear in the future. The conference was held May 24-25, 1976 in Bethesda, Maryland.

Project Title: PROCEEDINGS--INTERNATIONAL BLADDER STONE CONFERENCE, BANGKOK, THAILAND

Professional Personnel: Dr. G. Donald Whedon
Mrs. Billie B. Mackey

Project Description:

The 1972 International Bladder Stone Symposium was held in Bangkok, Thailand. The papers presented at this conference are being edited by the Chairman of the Symposium, Dr. Robert Van Reen, University of Hawaii at Manoa. The publication will be through the Government Printing Office and will be available in the fall.

Project Title: PROCEEDINGS--THIRD INTERNATIONAL CONFERENCE ON BONE MINERAL MEASUREMENT

Professional Personnel: Dr. G. Donald Whedon
Mrs. Billie B. Mackey

Project Description:

These proceedings are the third of a series on the subject of bone measurement sponsored by the University of Wisconsin Bone Mineral Laboratory. The first proceedings, METHODS OF BONE MINERAL MEASUREMENT, reported on the meeting of May 22-23, 1970. The second proceedings, INTERNATIONAL CONFERENCE ON BONE MINERAL MEASUREMENT, reported on a similar meeting held on October 12-13, 1973, at the Regency Hyatt House near the Chicago O'Hare Airport. These proceedings describe the Third International Bone Mineral Measurement Conference held in New Orleans, Louisiana on January 26-28, 1976.

The measurement of bone mineral content by photon absorption, an accepted research procedure and tool for clinical and biomedical investigations, has not been without problems. This meeting addressed difficulties concerning calibration, choice of instrumentation and measuring sites as well as interpretation of results. Newer developments such as the use of dual photon absorption, Compton scattering and neutron activation were also covered. It is through conferences such as the Third International Conference on Bone Mineral Measurement that interested researchers will facilitate resolution of these problems and aid in the incorporation of new perspectives. These proceedings will be published in the AMERICAN JOURNAL OF ROENTGENOLOGY.

Project Title: Second Annual Meeting of the American Association for Clinical Histocompatibility Testing

Professional Personnel: Dr. G. Donald Whedon
Mrs. Billie B. Mackey

Project Description:

This Second Annual Meeting was held in New Orleans, Louisiana. Histocompatibility testing is increasing in importance for organ transplants, blood transfusions, blood transfusion therapy, and it is now implicated in a multitude of chronic diseases such as rheumatic diseases, psoriasis, and juvenile-onset diabetes. Its use as a diagnostic tool for these chronic illnesses will have a significant impact on early diagnosis and treatment of these illnesses. The purpose of this meeting was to bring together the leading researchers and clinicians in histocompatibility testing, to assess its current state of knowledge, and to make recommendations for future research and use of the testing. The sessions covered such topics as B cell studies and HLA genetics, serologic and biochemical studies of HLA; HLA and transplantation, HLA and disease, and immune response and MLC studies. The proceedings will be published in the TRANSPLANTATION PROCEEDINGS.
The conference was held April 27-29, 1976.

Project Title: Study for Evaluation of Testing for Cystic Fibrosis

Professional Personnel: Dr. G. Donald Whedon
Dr. George T. Brooks
Mrs. Billie B. Mackey

Project Description:

This study was a cooperative effort by the National Academy of Sciences, the National Heart and Lung Institute, and the National Institute of Arthritis, Metabolism, and Digestive Diseases. The Committee for a Study for Evaluation of Testing for Cystic Fibrosis was established in 1974 to (1) assess reliability of the data on cystic fibrosis "factors" in serum and exocrine secretions and in cell culture, (2) evaluate the role of the cystic fibrosis "factors" in the detection of the heterozygote and homozygote, (3) evaluate the various screening tests for the diagnosis of cystic fibrosis in the newborn, and (4) evaluate the sweat test. The outcome of this evaluation was a Report of the Committee for a Study for Evaluation of Testing for Cystic Fibrosis and was published in the JOURNAL OF PEDIATRICS.

Project Title: Symposium and Proceedings on the Development of Iron Chelators for Clinical Use

Professional Personnel: Miss Merilyn C. Hiller
Mrs. Billie B. Mackey

Project Description:

This symposium was held September 22, 1975 and was the second conference on iron chelation. The first iron chelation conference was held September, 1974 to determine the status of research prior to awarding contracts. This second conference on iron chelation was held in order to have the contractors in the program as well as special consultants join together in exploring the problems encountered in designing more effective ways to chelate iron, and to assess the progress of the program. Topics covered included development and clinical use of iron chelators, isolation of microbial iron chelators, and the development and characterization of desferal. The proceedings of this Symposium will be published through the Government Printing Office and will be available in the summer.

Project Title: Workshop on Manpower and Research Funding in Dermatology

Professional Personnel: Dr. Laurence Miller
Mrs. Billie B. Mackey

Project Description:

This workshop was convened on February 20, 1976 in Bethesda, Maryland to assess the research and manpower needs through determining the current status of skin research. Assessments of future needs and priorities in basic, clinical and applied research were made as well as future manpower needs assessed.

Recommendations based upon these assessments were made.

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 years provide detailed background on the problems of end-stage kidney disease and elaborate on developments in the 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 develop- of improved and less expenses dialysis hardware and methodologies and at optimal rehabilitation of chronic dialysis patients. 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. In recent years 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 70 research and development projects are supported by contracts with universities, nonprofit and industrial research laboratories to give a broad approach to problems of highest priority. Projects are focused on problem areas including clinical trials of more efficient or less costly dialyzers, development of special adsorbents for uremic toxins, and studies to elucidate the nature of the aberrant metabolic changes in uremia.

The Program is also funding studies aimed at isolation and identification of toxic factors which must be removed from the patient's blood, in addition to metabolic wastes and other substances known to accumulate during kidney failure. A large number of research projects is also devoted to the pathophysiology of uremia in an effort to prevent or control complications in patients who have lost their kidney function and are being maintained with the aid of artificial kidneys. Much of the future success in simplifying the treatment of patients with end-stage kidney disease depends on developments in these and other areas of fundamental research.

Accomplishments

New Wearable Artificial Kidney

Scientists at the University of Utah, supported by a contract from the Artificial Kidney Program, have developed a wearable artificial kidney which may be available for general use within a number of years. The seven-pound device will be a welcome and needed addition to the various types of artificial kidneys currently available. The unit can be produced at a considerably lower cost than conventional dialysis machines, and it promises to make dialysis less expensive and possibly less damaging to the patient's red blood cells. It will also provide the great advantage of freedom of movement to users who presently must spend hours connected to large, stationary machines during conventional dialysis treatment.

According to Dr. Robert Stephen of the home dialysis training center at the University of Utah, where the device was developed, a patient using the portable unit would undergo dialysis every day instead of three times weekly, thereby maintaining a more even chemical balance in his blood and tissues and preventing a buildup of waste products. In its present stage of development, the portable artificial kidney cannot remove urea, a major waste product, from the blood. Therefore, in addition to using his portable unit two hours a day while moving about freely, the patient will spend one hour with the unit attached to a stationary 20 liter tank with dialysate (blood-washing solution) to remove urea.

Research Advances Related to Peritoneal Dialysis

Peritoneal dialysis, a method of abstracting impurities from the blood by utilizing the lining of the abdominal cavity, or peritoneum, and its blood supply as a dialyzing cavity, is at a stage that it can now be utilized for chronic maintenance therapy either in the home or in an institutional setting. New approaches to peritoneal dialysis are currently under study which ultimately may result in smaller and more efficient equipment for this mode of treatment, and in less complicated and costly treatment.

Dr. Christopher R. Blagg and associates at the Northwest Kidney Center in Seattle, Washington, have conducted a comparative study of home peritoneal dialysis with home hemodialysis in comparable patients under the care of a community dialysis program. His experience to date has demonstrated that the cost of treatment with home peritoneal dialysis is essentially similar to that of home hemodialysis with reuse of the dialyzer and is lower than hemodialysis if in the latter method the disposable dialyzer is not reused. The rate of hospital-based, emergency back-up dialysis for patients on home pertitoneal dialysis is somewhat higher than the rate for home hemodialysis patients, partly because of technical problems with the new automated peritoneal dialysis equipment, and also because, as a result of patient selection, the peritoneal dialysis patients generally tended to have more medical problems. No new major problems or complications with peritoneal dialysis were noted.

Dr. William J. Kolff and associates of the University of Utah School of Medicine have developed a sophisticated new access device to the peritoneal cavity. This experimental device permits a continuous flushing of the peritoneal cavity with dialysate (in contrast to the conventional episodic filling and emptying of the abdominal space with a dialysate solution). Preliminary results have shown that this new approach enhances the clearance of creatinine (one of the uremic waste products which must be removed) via the peritoneal route.

Dr. John F. Maher and associates at the University of Connecticut Health Center, and Dr. Karl D. Nolph and associates of the University of Missouri, have obtained preliminary experimental results that indicated that the clearance of some uremic wastes by way of peritoneal dialysis can be improved significantly by the systemic or intraperitoneal administration of certain vaso-active drugs. These studies are based on the assumption that peritoneal clearance may be increased if the blood capillaries which line the peritoneal cavity can be expanded (or possibly even be made more permeable) with the aid of specific drugs generally known to dilate capillaries or to increase their permeability.

Vitamin B_6 Nutrition in Dialysis Patients

The dietary requirement for vitamin B_6 in maintenance dialysis had not been established heretofore; chronic uremic patients have been reported to be deficient in vitamin B_6. Dr. Joel D. Kopple and associates of the University of California, Los Angeles Schools of Medicine and Public Health, have now demonstrated that a majority of patients on

maintenance dialysis who ingest the normal amount of approximately 1 mg. per day of vitamin B_6 in food will be deficient in this vitamin. A daily supplementation of 4 mg. of vitamin B_6 corrects this deficiency.

Trace Element Abnormalities

Dr. Allen C. Alfrey and associates of the University of Colorado School of Medicine, have been studying the composition of tissues of maintenance dialysis patients with respect to the concentrations of trace mineral elements which they contain, and have compared these values with concentrations found in normal subjects. They have found a significantly increased concentration of aluminum in the heart, bone, brain, liver, and skeletal muscle of dialysis patients. The most likely source of the increased body burden of aluminum would appear to be the aluminum containing phosphate-binding gels routinely administered to dialyzed uremic patients since other environmental sources of aluminum have not been identified. Animal studies are in progress to evaluate better the effect of orally administered aluminum-containing phosphate-binding gels on body aluminum burdens. These studies are of particular interest in connection with the possible causation of dialysis-asociated central nervous syndrome which leads to a characteristic speech defect, convulsive seizures, dementia and eventually death. Scientists studying this encephalopathy suspect that it might be caused by an abnormal accumulation of some mineral trace element in the brain and thus far the only consistent and most significant abnormality found in such patients is an increased aluminum content of the brain's gray matter.

Protein Requirements of Patients on Maintenance Dialysis

Dr. Frank A. Gotch and associate of the Franklin Hospital in San Francisco have conducted nitrogen balance studies in maintenance dialysis patients who were given diets containing different levels of protein. They have found that patients maintained on a low protein diet which supplies 0.5 gm. protein per kg. body weight were in negative nitrogen balance; thus this intake is not adequate for chronic dialysis patients despite the fact that this level of protein intake is generally considered adequate for normal nutrition in non-uremic individuals. Patients with intermediate protein intakes (1 gm./Kg.), on the other hand, maintain their nitrogen balance. Based on the day-to-day data obtained in these studies, the dialysis process itself apparently increases protein catabolism on the day of dialysis. The reason(s) for this are obscure at present and warrant further investigation.

Blood Cholesterol Levels in Treatment with Oral Sorbents

Dr. Eli A. Friedman and associates of the Downstate Medical Center in Brooklyn, are studying the use of orally administered oxystarch and activated charcoal in reducing the total body burden of uremic waste products. The principle involved is that these waste products will be selectively absorbed and bound to these sorbents in the intestinal tract (to which they migrate by way of the capillaries lining the gut), and will be carried out through fecal elimination. In continuing experiments they have shown that such a treatment decreases the blood-urea-nitrogen level of chronically uremic patients. There is a simultaneous increase in fecal nitrogen excretion during treatment with oxystarch, but only insignificant further increases in fecal nitrogen excretion were noted during combined treatment with oxystarch and charcoal. An interesting serendipitous finding was noted during these studies; mean serum cholesterol fell in the group from 200 mg% to 166 mg% on the average, and in each patient, during combined therapy with oxystarch and charcoal. It was concluded that, in addition to the previously noted increased nitrogen excretion during oxystarch treatment, the reduction in serum cholesterol observed in this oxystarch-and-charcoal trial may have clinical application in uremic and non-uremic patients in connection with future attempts to diminish blood cholesterol levels to retard atheromatous blood vessel degeneration.

Contractor: Baylor College of Medicine

Amount: $117,877

Title: Abnormalities of Carbohydrate Metabolism in Chronic Uremia

Objectives: The proposed work focuses on three major aspects of carbohydrate metabolism, including the role of inappropriately excessive or inadequate gluconeogenesis, the regulation of muscle protein degradation in the supply of gluconeogenic amino acids, and the extent of suppressibility of endogenous glucose production by exogenous dialysate glucose.

Major Findings: There is increased alanine production and release from skeletal muscle in uremia and this increase derives in part from an apparent direct effect of parathyroid hormone on skeletal muscle protein degradation. The utilization of amino acid carbon for muscle protein resynthesis in uremia is reduced substantially owing perhaps to primary alterations of Vitamin D metabolism in uremia. As a result, there is an increased rate of total glucose production and of gluconeogenesis from alanine in patients with chronic uremia, which derives in part from an increased alanine delivery rate from peripheral tissues, and in part from a redirection of hepatic alanine metabolism.

Proposed Course: The rate of endogenous glucose production will be determined by isotope dilution in uremic patients prior to and immediately after dialysis, in normal controls, and in patients with moderately severe renal failure not requiring dialysis.

Protein turnover will be estimated using dual label amino acid incorporation studies with acrylamide gel electrophoresis to separate the individual protein moieties. The kinetics of endogenous glucose utilization and production as well as the appearance and disappearance of dialysate glucose will be determined simultaneously in uremic patients using a double isotope protocol.

Contractor: Boston University

Amount: $75,914

Title: Mechanism of Hyperlipidemia in Chronic Renal Failure

Objective: To understand the lipid abnormalities that occur in chronic renal failure and to provide a basis for the treatment of these complications.

Major Findings: Uremia was produced in 80-100 g male Sprague-Dawley rats by ligation of the secondary branches of one renal artery, followed in one week by contralateral nephrectomy. The blood urea nitrogen concentrations rose to over 100 mg % (normal: 20 mg %); the rats gained less weight (4.0 g/day) as compared to sham-operated and unoperated animals (6.5 g/day).

After one month of renal failure, the rats were placed on a lipogenic diet for 10 days. Twenty-four rats were then sacrificed and their livers perfused in an isolated perfusion chamber with Krebs-Ringer bicarbonate buffer, 4% albumin, 25 mM glucose, and tritiated water. The incorporation of tritium into long chain fatty acids and B-OH sterols was determined. There was a significant, 2.5 fold inrease in fatty acid synthesis in the uremic livers, compared to the sham-operated animals. The increase in fatty acid synthesis was similar when the uremic livers were perfused with 5 mM glucose, B-OH sterol synthesis was likewise increased 2 fold in the perfused uremic liver, compared to sham-operated animals. There was no significant difference in the weight of the livers in the animals; thus the total amounts of hepatic fatty acids and B-OH sterols produced was elevated in the uremic animals.

The results indicate that the liver plays a role in the lipid abnormalities that occur in the rat with uremia. Present studies are assessing the rates of gluconeo-genesis and ketogenesis in the liver during chronic renal failure.

Proposed Course: The nature of altered lipogenesis in Uremia will be examined using cross-over experiments (normal livers and livers from uremic animals will be perfused with blood from normal animals).

Contractor: University of California

Amount: $61,790

Title: Evaluation of Chronic Peritoneal Dialysis in Children: Comparison with Hemodialysis.

Objectives: To compare peritoneal and hemodialysis effectiveness in children. The parameters to be followed are:
survival, dialysis access, biochemical control, convulsions, anemia, hepatitis, hyperlipidemia, hypertension, peripheral neropathy, osteodystrophy, growth and nutrition, sexual maturation, psychosocial adaptation, cost.

Major Findings: New Contract.

Contractor: University of California

Amount: $16,000

Title: Trace Element Abnormalities in Chronic Uremia

Objectives: To determine the levels of concentration of trace elements in dialyzed and non-dialyzed uremic patients; to determine the clinical importance of trace element abnormalities, and to establish the concentration of trace elements required in the dialysate solution.

Major Findings: The contractor has completed all the analysis in hair, RBC, plasma and urine by emission spectroscopy on fifty (50) normal control subjects and twenty (20) non-dialyzed uremic patients.

Proposed Course: Studies will be continued to include analysis of hair. RBC, plasma, urine and dialysate of uremic patients following dialysis therapy.

Contractor: University of California, Los Angeles

Amount: $89,815

Title: Nitrogen Metabolism in Uremia

Objectives: To study the metabolism of amino acids and their products and the enzymes which mediate the metabolism of these substances in uremia. It is believed that this information may help to define cause of uremic wasting and uremic toxicity, and may provide means of assessing optimal dietary treatment.

Major Findings: Histidine has previously been shown by the investigator to be an essential amino acid. In this contract year the investigator has further defined the daily histidine requirement. The daily vitamin B_6 requirement has also been further defined in dialysis patients. It was also found that phenylalanine and tyrosine metabolism is abnormal in uremic patients.

Proposed Course: The ongoing studies will be continued. Dietary histidine requirements will continue to be assessed. The metabolic effect of diets providing protein or essential and non-essential amino acids in patients with acute and chronic renal failure will be studied. Platelet serotonin metabolism and amine metabolism in chronically uremic patients will be evaluated and tissue amino acid levels will be studied in patients undergoing maintenance hemodialysis.

Contractor: University of California at Los Angeles

Amount: $105,000

Title: Controlled Evaluation of Maintenance Peritoneal Dialysis

Objective: The study has two objectives: (1) To evaluate the severity and causes of malnutrition and wasting in patients undergoing long-term peritoneal dialysis, it is proposed to evaluate nutritional status in a controlled study of uremic patients randomly assigned to treatment with either hemodialysis or peritoneal dialysis. The patients will be participating in an independently funded study funded by the Veterans Administration. (2) To evaluate the relative clinical outcomes of diabetic patients assigned either to hemodialysis or peritoneal dialysis, it is proposed that prospective, controlled comparison of home peritoneal and home hemodialysis be carried out in diabetic patients who are not a part of an independent VA cooperative study.

Major Findings: Metabolic studies are being initiated to characterize protein requirements in patients undergoing maintenance peritoneal dialysis. Such studies are designed to evaluate both the effect of varying protein intakes and the frequency of dialysis therapy on nitrogen balance and the generation rates and plasma levels of nitrogenous metabolites.

Studies have been undertaken to evaluate the glucose load absorbed during peritoneal dialysis and the necessity for additional insulin. Interperitoneal insulin has been proposed for the management of hyperglycemia developing during peritoneal dialysis in diabetic patients. A canine model for peritoneal dialysis has been devised to evaluate the use of interperitoneal (IP) insulin during dialysis. With this technique, the trans-peritoneal kinetics of insulin transfer during peritoneal dialysis and its biologic effects were evaluated in dogs undergoing peritoneal dialysis. Changes in plasma glucose and immunoreactive insulin (IRI) during dialysis with 10, 20 and 30 U/insulin/L dialysate and during the intervenous (IV) infusions of insulin at 0.5 or 1.5 U/hr were measured. Also, recovery of

^{125}I-insulin added to dialysate was evaluated. The present studies suggest that interperitoneal insulin should be used cautiously and in low concentration because of possible cumulative and late effects.

Proposed Course: This project will be continued.

Contractor: University of California, San Diego

Amount: $148,5000

Title: Blood Pressure Control with Hemodiafiltration

Objectives: To determine if hemodiafiltration can be used to control significant hypertension in chronic renal failure patients.

Major Findings:

1. Three patients with chronic renal failure maintained with hemodiafiltration as the sole method of maintenance showed no sign of depletional syndromes for a period of up to 1 year in one patient and 6 and 3 months respectively in the other two. The degree of "well being" when compared with hemodialysis was considered to fall in the upper 15% of our experience for maintenance hemodialysis in two and about average in the third.

2. The objective measurements made on blood chemistries, (BUN, creatinine, uric acid, phosphate calcium, CO_2 cont, PH) during maintenance diafiltration showed greater interpatient variability than difference from the control measurements made during maintenance hemodialysis.

3. Serial clinical examinations showed marked improvement in cardiovascular status in all three patients studied. Specifically, blood pressure, after stopping antihypertensive medication, returned to normal (defined as a pre-treatment blood pressure of 110-120/70-80) in one and to near normal (125-150/70-80 and 150-160/95-100) in the other two in spite of continued episodic fluid overloads due to dietary indiscretion. Radiographic evidence of decrease in heart size was present in two at the end of 3 months and was unchanged from normal in the third.

Proposed Course:

The present clinical protocol involves a 3 month crossover study in which patients maintained with conventional hemodialysis, who have significant hypertension, have blood pressure, heart size and e.k.g. recorded. In addition, using radioisotope dilution techniques total body water, extracellular and intravascular volumes will be measured. Total body sodium will also be measured. Where possible, cardial output and peripheral resistance will be measured using non-invasive ultrasonic techniques. After 3 months on maintenance hemodiafiltration these measurements will be repeated. A third set will be obtained as a bracketing control period. Single and multiple correlations will be sought between treatment modality and blood pressure, heart size, total body water and sodium, vascular volume, cardiac output and peripheral resistance.

Contractor: University of California at San Francisco

Amount: $81,086

Title: Mechanism of Hyperlipidemia in Chronic Renal Failure

Objective: Extensive lipoprotein and peptide studies will be performed on anephric patients on dialysis, on patients with glomerulonephritis, on patients with polycystic disease, and on normal control subjects. These studies will help to define the underlying mechanisms of the hyperlipidemia of chronic renal failure and may form the basis for therapeutic measures which may alleviate the condition.

Major Findings: Analysis of the lipoprotein spectrum of uremic patients has revealed some interesting correlations. Fasting plasma samples taken immediately before heparinization and dialysis were compared with fasting plasma samples taken from normal control subjects. The results are as follows: Very Low Density Lipoprotein (VLDL) Triglycerides were significantly elevated in anephric patients ($p < .001$). Low Density Lipoprotein (LDL) cholesterol was significantly lower in the patients ($p < .01$). Both High Density Lipoproteins (HDL) cholesterol and protein were markedly lower in the patients ($p < .001$). There was an excellent negative correlation between the level of HDL cholesterol ($r = -.86$) and HDL protein ($r = -.81$) and the VLDL triglycerides. Since the HDL serve as an activator for VLDL removal by lipoprotein lipase and since LDL is a direct product of VLDL metabolism, these data clearly point to a defect in the removal mechanism for VLDL. In preliminary studies the composition of HDL is not different from normal although the plasma content is lower. The VLDL show quite different results. The ratio of activator peptide (Apo C-II)/inhibitor peptides (Apo C-III) is markedly lower in the patients so far studied. This ratio shift may have a marked effect on the lipoprotein lipase reaction.

Proposed Course: A quantitative estimation of the apoproteins in each of the lipoprotein species will be determined by polyacrylamide electrophoresis. Particular attention will be given to quantitating the C-II and C-III peptides of HDL and the VLDL spectrum.

Determination of "activator" or "inhibitor" potential of whole plasma and of VLDL and HDL apoproteins will be carried out.

Techniques will be investigated for obtaining C-II peptides from whole serum by a heparin-divalent cation technique. Preliminary investigations of this fraction for replacement therapy will be carried out.

Contractor: The Regents of the University of California, San Francisco

Amount: $ 71,666

Title: Abnormalities of Nervous System Function in Uremia

Objective: To characterize those biochemical alterations of the central nervous system, peripheral nerve and skeletal muscle which are present in both acute and chronic renal failure and to determine how these alterations are affected by hemodialysis.

Major Findings: It was found that in animals with acute renal failure, brain Ca^{++} was significantly elevated. This increase was prevented by parathyroidectomy (PTX). Administration of parathyroid extract (PTE) to normal or PTX-uremic animals increased brain Ca^{++}. The elevated brain Ca^{++} was found to be caused by a direct effect of parathyroid hormone (PTH) on brain Ca^{++} transport.

During rapid hemodialysis, the pH of CSF was observed to fall as blood pH rose. The decline in pH of CSF may be related to a fall in brain intracellular pH (pHi). To investigate such a hypothesis, a method to measure brain pHi was developed, employing a double isotope technique ($C^{14}DMO$, $^{35}SO_4$). Brain pHi increased during acute respiratory alkalosis, and fell during acute respiratory acidosis. During respiratory acidosis, brain pHi gradually rose to normal secondary to increased production of HCO_3^- by the brain. During respiratory alkalosis, brain pHI gradually fell to normal secondary to increased brain lactate. In all animals, movement of DMO into brain cells was shown to be pH dependent. These findings appear to establish the validity of the method for evaluation of changes in brain pHi.

Proposed Course: During the forthcoming year the investigators will continue to evaluate the changes in the central nervous system in animals with chronic renal failure and study the effect of hemodialysis on these changes. Brain intracellular pH will be studied during development of uremia and therapy with slow or rapid hemodialysis and dialysis against dialysate containing mannitol or glycerol. Change in peripheral nerves during acute uremia in animals will also be evaluated.

Contractor: University of Southern California

Amount: $84,771

Title: Osteodystrophy and Divalent Ions in Kidney Failure

Objective: To carry out studies to evaluate various aspects of deranged divalent ion metabolism in renal failure and to evaluate the effectiveness of $1,25(OH)_2D_3$ in correcting the abnormalities of divalent ion metabolism.

Major Findings:

1. One of the mechanisms that underlie the hypocalcemia of renal failure is skeletal resistance to the calcemic action of PTH. This abnormality has been documented in patients with acute renal failure, those with mild and advanced renal failure and in dialysis patients. A deficiency of $1,25(OH)_2D_3$ is, at least partly, responsible for this abnormality.

2. Intestinal absorption of calcium is defective in patients with advanced renal failure. The defect is probably located in duodenum and early jejunum where vitamin D effects calcium absorption. Administration of $1,25(OH)_2D_3$ can correct the defect.

3. Preliminary studies indicate that long-term therapy with 1,25 $(OH)_2D_3$ may improve the bone lesion due to hyperparathyroidism in patients with renal failure.

Proposed Course: The major goals of this project are nearly completed. During the forthcoming year the investigators will continue studies to evaluate various aspects of deranged divalent ion metabolism in renal failure in an effort to understand their mechanisms and seek rational approaches to their prevention and management. This proposal is based on the information gathered in the last seven years through research supported by contracts with the Chronic Uremia Program. Four aspects of the deranged divalent ion metabolism in renal failure will be studied. These include 1) evaluation of the mechanisms of the hypocalcemia and apparent skeletal resistance to parathyroid hormone, 2) studies of intestinal absorption of calcium in renal failure, 3) evaluation of bone biopsies in patients with early and moderate renal failure, and 4) evaluation of the effect of $1,25(OH)_2D_3$, and 1 OH D_3.

Contractor: Cedar-Sinai Medical Center

Amount: $104,825

Title: Sorbent-based Regenerative Peritoneal Dialysis System

Objectives: To develop a peritoneal dialysis system in which the dialysate is circulated through a sorbent cartridge for regeneration.

Major Findings: A prototype equipment has been tested in human subjects for sterility, toxicity, effectiveness of the filtration system, biochemical studies and clinical results. Another prototype operating under continous flow was tested with animals with encouraging results.

Proposed Course: Four chronic patients will be maintained solely on sorbent peritoneal dialysis. Between twenty (20) to forty (40) acute peritoneal dialysis patients will be treated with this modality of dialysis. Continuous flow peritoneal dialysis will be tested in human subjects.

Contractor: Celanese Research Company

Amount: $100,000

Title: Preparation and Evaluation of Optimized Hemodialysis Membrane

Objectives: To prepare superior membranes for hemodialysis from cellulose acetate and other polymers with emphasis being placed on practical results. The program is divided into three distinct phases: production (casting maching improvements, low hydraulic permeability CA membranes, membrane supply for clinical trials, intermediate hydraulic permeability membranes); development (optimization of membrane geometry/transport properties, PBI membranes; and some limited exploratory work.

Major Findings: This program has generated two distinct membrane variants CA-1 and CA-2. Both have been well characterized and a supply of each adequate for clinical studies, is on hand. CA-1 and CA-2 are both cellulose acetate films, cast from an acetone/formamide solvent and coagulated in water. Their divergent properties were achieved through careful control of the casting solvent ratio and drying conditions. Membranes possessing hydraulic permeabilities anywhere from zero to several hundred times Cuprophan PT-150 can be achieved by this technique. Ca-1 and Ca-2 are examples of clinically useful membranes which have been fabricated in this manner, but many other balances of properties are achievable.

Low MW cutoff membranes have been prepared on a limited scale by treating CA-1 with methanol/toluene mixtures of carefully controlled composition under well-regulated conditions of shrinkage. Scale-up procedures are currently being developed. The feasibility of fabricating thinner versions of CA-1 and CA-2 and a high flux membrane superior to Rhone-Poulenc's PAN membrane has likewise been demonstrated in the laboratory.

In the area of PBI membranes, blood compatibility determinations on variously heparinized PBI membrane samples are now in process and two prototype hollow fiber dialyzers have been constructed.

Proposed Course: Added emphasis will be placed on obtaining evaluation data from cooperating clinicians. The possibility of making a low cut-off experimental membrane will be considered.

Contractor: University of Colorado

Amount: $121,936

Title: Trace Element Abnormalities in Chronic Uremia

Objectives: To determine the levels of concentration of trace elements in dialyzed uremic patients; to determine the clinical importance of trace element abnormalities, and to establish the concentration of trace elements required in the dialysate solution.

Major Findings: Considerable progress has been made in improving analytical techniques and tissue preparation for analysis. Plasma trace element constituents have been more fully characterized in control subjects. Preliminary studies have been carried out on dialysis patients in other geographical areas. Studies have been conducted to further characterize the body burden of a number of trace elements.

Proposed Course: Studies will be continued using three methods of analysis. The proposed studies will attempt to determine:

(1) Tissue trace element profiles in chronic uremia
(2) Contaminants in dialysis equipment
(3) Transfer through membranes
(4) Geographical variations
(5) Trace element excretion in uremia
(6) Effect of duration of uremia on trace element levels
(7) Studies in some specific trace elements: Rubidium, Aluminum and Tin
(8) Special animal studies
(9) Epidemiological studies of the Dialysis Dyspraxia Syndrome

Contractor: University of Connecticut

Amount: $50,465

Title: Peritoneal Permeability and Drug Enhancement in Uremia

Objectives: Comparison of clearance values of solutes of varied size before and after vaso-dilator enhancement will allow characterization of peritoneal permeability, will quantify peritoneal clearances of numerous drugs and will identify quantitatively the beneficial effect of pharmacologic manipulations.

Major Findings: Because Food and Drug Administration approval has not been granted for administration of standard drugs for an unapproved purpose, the study has been limited mostly to determination of baseline clearances. The measured creatinine and urea clearances in the normal, 16.3 +/-1.3 and 20.1+/-1.4 and creatinine/urea clearance ratio of 0.81 is in accord with data published. In one patient with vascular disease, clearances of 9.3 and 14.0, ratio .66, increased after clinically indicated dipyridamole given systemically to 13.3 and 19.7 respectively with a ratio 0.68. These preliminary data are in accord with the hypothesis. The results are as yet too preliminary to quantify clearances or derive permeability data. The early phases of the study have rather involved establishment of the GLC-MS methodology for solutes of particular interest.

Proposed Course: The investigators have begun to study available and willing patients undergoing clinically indicated peritoneal dialysis who are hemodynamically stable without infection and who can be classified by histologic or clinical data into presence or absence of vascular disease. Dialyses are performed with a 10-minute infusion, 30-minute dwell and 30-minute drainage of dialysate of standard isosmotic composition. The study commences after a satisfactory free flow equilibrium is reached. After measurement of clearances of routinely measured solutes like urea and creatinine as well as a spectrum of larger solutes of endogenous or exogenous origin identified by gas-liquid chromatography-mass spectrometry-computerized analysis, vasoactive drugs are administered systemically or intraperitoneally and three additional clearances are determined. When possible, three control clearances are followed by three clearances with intraperitoneal infusion of a test drug. Systemic administration of the test drug is then instituted and three more clearances measured on the following day. Comparison of clearances and clearance ratios before and after the administration of the test drugs allows determination of the effect on permeability from either surface of the membrane.

The use of GLC-MS analysis is being undertaken to identify clearances of exogenous and endogenous solutes in peritoneal fluid.

CONTRACTOR: State University of New York, Downstate Medical Center

AMOUNT: $58,190

TITLE: Clinical Efficacy of Oxidized Starch and Charcoal in Uremia

OBJECTIVE: A controlled trial of oral oxidized starch and charcoal in uremic patients undergoing maintenance hemodialysis will be initiated. After defining the chemical status and response to dialysis in thrice weekly dialyzed patients, a reduction in dialysis frequency to once weekly will be attempted during treatment with both sorbents. Should a one month trial of sorbents be well tolerated, an extension of therapy will be considered upon review of all chemical results.

MAJOR FINDINGS: A controlled trial of Miles Sumstar 190 oxidized starch and charcoal was completed in four hospitalized uremic patients in a clinical research center. As had previously been found for Italian (Giordano) oxystarch, a significant increase in mean fecal nitrogen excretion (from a control of 1.5g/24 hr. to 3.1g/24 hr.) and mean fecal potassium excretion (from a control of 21.8 mEq/24 hr. to 43 mEq/hr.) was observed during oxystarch ingestion. Decreases in urinary nitrogen and potassium excretion during oxystarch treatment maintained neutral nitrogen and potassium balance.

Gavage feeding of oxystarch and charcoal to binephrectomized rats indicated that sorbents can prolong life from a control mean of 3.0 days to 6.0 days indicating the potential clinical value of sorbents in uremia.

PROPOSED COURSE: The proposed study will be completed during the current contract year.

Contractor: Exxon Research Engineering Company

Amount: $148,420

Title: Liquid Membrane Capsule System for the Treatment of Chronic Uremia

Objectives: To develop a liquid membrane to encapsulate reactants to be used as toxin traps in the treatment of chronic uremia.

Major Findings: Ammonia removal rate has been improved several fold. Organic acids have been encapsulated. Urease has been encapsulated. Stability of the LMC has been improved. Extensive animal experiments have been conducted with liquid membrane capsules to determine *in vivo* transfer rates and tolerance.

Proposed Course: Research will be conducted to further improve the stability of the LMC in the presence of bile and improve its transfer rate and capacity. Further animal tests will be performed to determine *in vivo* performance of the improved LMC.

Contractor: Franklin Hospital

Amount: $98,865

Title: Solute Kinetics in Intermittant Dialysis Therapy

Objectives: The object of these studies is to investigate the mechanisms that control the concentrations of various solutes found in the chronic uremic and how they can be related to the patient's state of health. Proposed work concentrates, therefore, on the kinetics of nitrogen based protein catabolites (urea, creatinine, polypeptides) and the body water buffer system. The results of these studies are anticipated to provide new information about pathophysiologic mechanisms operative in dialysis and a data base for quantitative prescription and control of this treatment. In the forthcoming year a comprehensive model of hydrogen ion metabolism in dialysis patients will be developed, feasibility modeling studies of anemia in uremia will be carried out, kinetic control of urea will be evaluated and additonal nitrogen balance and metabolic studies will be completed.

Major Findings: Recent results are: (1) implementation of kinetic modeling based on urea metabolism with 61 patients currently modeled at an average treatment time of 11 hours. The major influence on accuracy of therapy prediction is the constancy of G. (2) Patient response to modeled therapy has been exhaustively investigated. Factors examined were: Nutrition, ultrafiltration requirements and associated morbidity, potassium, acid-base, phosphorous and calcium metabolism, anemia peripheral neuropathy, frequency and duration of hospitalization, rehabilitation, and cumulative survival. None of these factors was found to be adversely affected by individualization of therapy. (3) Five patients have been studied under controlled nitrogen metabolic conditions. These studies verified the clinical kinetics as an accurate method of V and G determinations; they also verified the PCR-G relationship. They revealed that dialysis may very well be a catabolic influence on dialysis patients. Patients on high and low protein diet were found to be relatively catabolic on dialysis days compared to off days. (4) Acetate kinetics were evaluated. Patients were found (a) to seldom reach steady state levels during 3-5 hour dialyses, and (b) to have saturated metabolic mechanisms (2.5-3.7 mEq/min) for this anion at blood levels found during dialysis. (5) Creatinine and uric acid were also studied, as were membranes and dialyzers.

Proposed Course: Current plans are to continue this definitive investigation.

Contractor: Gulf South Research Institute

Amount: $94,474

Title: Membranes and Materials Evaluation: Permeabilities, Physical and Mechanical Properties

Major Findings:

- Methods for characterizing the mechanical and mass transfer properties of hollow fibers were developed and evaluated. Three hollow fibers were characterized.

- The rotating cell (in use at GSRI) for transport measurements was compared to the Kaufmann-Leonard cell using the standard lot Curophan membrane. Results were not significantly different.

- New marker molecules were developed: two lipids and four polypeptides

Proposed Course:

Work on the marker molecules will be ccontinued. Trace elements content of membranes will be evaluated. Dialyzer performance will be evaluated. Solute rejection coefficients will be measured.

Contractor: Harbor General Hospital

Amount: $200,000

Title: Research in the Role of Acetate in Dialysate for Hemodialysis

Objective: To define the consequences of using acetate as a buffer in hemodialysis.

Major Findings: The data to date is limited. The studies on pedicle clamped animals indicate a generalized increase in free fatty acids with the acetate loads given. ^{14}C is incorporated into all fat fractions with the liver and aorta showing heaviest incorporation into phospholipids and triglycerides. It was stressed that the counts are low and that improvement in the counts could be obtained by either increasing the radioactive load or pooling tissues from increased numbers of experimental animals rather than studies on individual dogs.

Proposed Course: The animal studies call for the administration of measured amounts of acetate and radioactive acetate (^{14}C) to dogs during dialysis and measurement of radioactivity incorporated into CO_2 and into cholesterol, triglycerides, fatty acids and phospholipids in various tissues. The tissues being examined after sacrifice includes: heart, aorta, liver, spleen, sciatic nerve, omental fat, testicle, epidymal fat, serum and plasma. Unlabelled acetate levels administered during dialysis are determined by inflow/outflow differences on both the blood and hemodialyzer sides as determined by a GLC method for acetate. Labelled acetate is administered intravenously and the balance is determined by measurement of radioactivity in the dialysate. The human studies will examine the various lipid constituents of blood and tissues which were selected for accessibility as well as relevency. (red cells, leukocytes and subcutaneous adipose tissue) during prolonged dialysis with acetate containing and acetate free dialysate. The study will be conducted in a cross-over manner.

Contractor: Harvard University

Amount: $48,465

Objective: The study proposes to define the dialysis success profile (DSP) and measure it in a large group of patients. An attempt will be made to correlate dialysis success with measures of blood access success. The DSP is a quantitative three-part measure of dialysis efficacy. It is a composite of three factors: biochemical, disease-related, and psychosocial. Once defined and measured, it will permit group comparisons of patients by diagnostic category or by therapy schedule. DSP will be measured in a large stable group of chronic dialysis patients and the results correlated with patient, disease, and therapy-related factors. Particular attention will be given to correlation with details of access to the circulation. An hypothesis to be tested is that access efficacy, complications, and failures powerfully influence dialysis success.

Major Findings: The proposed dialysis success profile (DSP) has three components. The first is the biochemical and physiologic effect of dialysis. The second component of DSP is a record of symptoms and findings common to patients with end stage renal failure to provide a measure of the medical status of the patient. The third part of the DSP is an aggregation of psychological and social factors that express the result of the entire treatment experience in chronic hemodialysis.

Studies involving chart review and patient interviews, which are designed to define dialysis success in terms of morbidity, mortality and various estimators of social adjustment, have been completed. The population studied consist of those individuals who have received care for chronic renal failure from physicians at the Peter Bent Brigham Hospital currently and for the past 5 to 8 years. Independent variables such as morbidity and social adjustment were examined as functions of certain demographic characteristics (e.g., race, religion, education, socioeconomic status, etc.) and medical conditions (e.g., arteriosclerotic heart disease, hypertension, diabetics, age, basic renal disease, etc.) which were present at the institution of hemodialysis therapy.

Three separate but interrelated studies were designed and data collection has been completed. The three protocols, or sub-projects, are titled: 1) Medical Risk Factor Analysis, 2) Psychosocial Adjustment Studies, and 3) Problems Relating to Vascular Access in Dialysis Patients. In addition, a questionnaire to estimate the severity of various symptoms commonly found in dialysis patients has been developed and is under evaluation.

With respect to symptoms most patients were able to concentrate adequately and had a good appetite. Moderate fatigue and sleeplessness were common, however, as were pruritis and cramps. Moderate to severe sexual dysfunc-.

tion was almost universal. The questionnaire has been reconstructed to be shorter and clearer and is currently being administered to patients.

Data analysis in the Risk Factor study is very preliminary. However, of the 90 patients used in a pilot analysis, the incidence of cardiovascular risk factors at the institution of hemodialysis therapy is relatively high, and a history of hypertension is common. Further, within the first year, a substantial fraction of hospitalizations is spent for problems relating to vascular access.

Proposed Course: This project will be completed during the current year.

Contractor: Indiana University Foundation

Amount: $60,000

Title: Carbohydrate and Fat Metabolism in Uremia

Objectives: The major focus of this investigation is to examine the role of parathyroid hormone and secondary hyperparathyroidism in the pathogenesis of carbohydrate and lipid abnormalities in chronic uremia.

Major Findings: Results to date indicate that a) carbohydrate tolerance is impaired in the uremic dogs b) plasma glucose is higher after alanine infusion in the uremic dogs and c) plasma glucagon values are strikingly and abnormally high in uremic dogs. Preliminary results suggest that the plasma glucagon varies directly with the degree of renal failure. Studies of the effects of somatostatin are in progress.

Parathyroid hormone in pharmacologic doses increases gluconeogenesis and glycogenolysis in isolated rat hepatocytes. However, in the dog, parathyrdid hormone does not increase the blood sugar in vivo and does not activate adenylate cyclase in hepatic membranes in vitro. Thus, secondary hyperparathyroidism cannot augment blood glucose by any hepatic effects in this species.

Proposed Course: Fasting serum calcium, phosphorus, urea nitrogen, insulin, glucagon, PTH, and glucose will be determined in normal adult volunteers as well as in patients with chronic renal failure. Alanine and glucagon infusions will also be performed. The effects of repeated doses of PTH on plasma free fatty acids and fasting blood glucose will also be studied. The effects of highly purified bovine parathyroid hormone on the release of insulin and glucagon by perfused isolated rat pancreatic islet cells will be determined.

Contractor: Jefferson Medical College

Amount: $43,583

Title: Amine Metabolism in Uremia

Objective: To study the metabolism and toxicology of aliphatic amines in uremia with emphasis on correlating methylamine levels with uremic symptomatology.

Major Findings: The abnormal bacterial flora within the small bowel of uremic patients has been shown to generate trimethylamine. The elevations of duodenal dimethyl amine and increased numbers of bacteria occur when the serum creatinine approaches 8 mg%. Oral administration of nonabsorbable antibiotics decreases the serum methylamine levels. In one patient there was striking improvement in central nervous system symptoms which recurred when antibiotics were discontinued. The methylamines are volatile and appear on the breath accounting in part for the fishy amine odor.

Proposed Course: The investigator has almost completed this project. The last six months will be spent studying the clinical course of uremic patients and correlating neurobehavioral parameters with methylamine levels. In addition, further studies of gastrointestinal tract flora will continue in order to determine how they are related to methylamine production.

Contractor: The Johns Hopkins University

Amount: $ 54,352

Title: Serum Lipid and Lipoprotein Abnormalities in Chronic Renal Failure

Objective: To assess the incidence and nature of hyperlipidemia in chronically uremic patients and to determine the effectiveness of clofibrate in lowering serum lipid levels during chronic hemodialysis and after renal transplantation.

Major Findings: Triglyceride determinations vary considerably in patients with chronic renal failure. It may not be possible to classify patients with chronic renal failure into normo- or hyperlipidemic sub-groups on the basis of a single triglyceride determination.

Twelve of twenty-six chronic dialysis patients had hypertriglyceridemia all with type IV hyperlipoproteinemia. No differences in dietary intake were found between normo and hyperlipidemic patients. Twelve of twenty renal transplant patients were hyperlipidemic. In chronic dialysis patients neither PHLA nor histaminase activity increased after heparin infusions. This suggests that heparin unresponsiveness as described in patients with dysproteinemia may also occur in chronic renal failure.

Proposed Course:

1. Determine the effectiveness of clofibrate in reducing serum lipid levels in hypertriglyceridemic patients
   a) During chronic hemodialysis and
   b) After renal transplantation

2. Perform longitudinal studies on the natural history of hyperlipidemia in patients with chronic renal failure before and during hemodialysis and after renal transplantation

3. Compare the composition and size of VLDL and intermediate density lipoprotein (d 1.006 to 1.019) in hypertriglyceridemic patients with familial type IV hyperlipoproteinemia, and normal subjects.

4. Measurement of plasma histaminase activity

Contractor: Karolinska Institutet

Amount: $69,698

Title: A Study of Uremic Toxicology

Objectives: Utilizing the technique of high speed gel filtration various uremic body fluids have been separated into 10 component ultraviolet absorbing peaks. Distinct and readily recognizable differences in these peaks have been detected when uremic plasma and urine are compared to normal plasma and urine. The method of gradient elution appears to have an accuracy permitting quantitative analysis. Subsequent studies will be directed at attempting to correlate the absolute and relative magnitude of the sub-peaks of peak 7 to uremic symptomatology and clinical conditions. Quantitative analysis of these peaks will be used in evaluation of the influence of dialyzer designs, membranes, etc., on the removal of naturally occurring middle molecules. Attempts will also be made to further separate, isolate and identify the middle molecule peptides which accumulate in uremia.

Major Findings: Methods were developed whereby middle molecules can be separated and quantitated in biological fluids. The methods involve a high speed gel filtration technique (HSGF) combined with gradient ion exchange chromatography (GEC). The solutes were detected at 254 and 206 nm. The molecular weight range of the fractions isolated by HSGF was assessed with standards of known molecular weight. The recovery of the methods (HSGF + GEC) was 92% and the coefficient of varation in the integrated area of a single middle molecule peak varied between 4.6 and 11% depending on the peak size.

By the HSGF method normal and uremic plasma and urine were separted into 10 or 11 peaks based upon differences in molecular size. One of these peaks, no 7, which was present in uremic plasma but not detected in non-uremic plasma contained middle molecules (mol wt 1,000-2,000). Amino acid analysis before and after acid hydrolysis in peak 7 material obtained by preparative technique, showed this peak to contain a mixture of peptides.

Using the GEC method peak 7 was further separated into 7-8 new peaks (7a, b, c, etc.). Plasma from six normal subjects and six non-uremic patients with various diseases yielded only two peaks, 7f and g, whereas urine from normal subjects and plasma and urine from uremic patients contained all or most of these peaks.

The relative concentration of the sub-peaks in plasma from patients with renal failure was evaluated from the integrated peak area of the chromatograms and plotted against the creatinine and urea-nitrogen in plasma. Measurable peaks were only found in patients with creatinine above 6 mg% and creatinine clearances below 10 ml/min.

The investigators have observed that uremic patients who are adequatedly dialyzed and are doing well have low middle molecule peaks and that the dialysis procedure further decreases most of these peaks, which appear in the dialysis fluid. On the other hand, patients who develop complications during dialysis treatment tend to accumulate middle molecule material.

Proposed Course: This project will be continued.

Contractor: Mayo Foundation

Amount: $79,620

Title: Uremic Neuropathy

Objectives: To develop devices to detect alterations in peripheral nerve function that may occur with changes in renal function. To assess adequacy of dialysis of these measures.

Major Findings: Touch pressure and pallesthesia instruments have been designed. Normal ranges for measurements with these devices have been established.

Proposed Course: The investigators will examine the usefulness of the touch pressure and pallesthesia instruments in assessing adequacy of dialysis therapy in chronic renal failure patients.

a) Touch pressure and pallesthesia measurements will be compared with clinical symptomatology, physical examination and nerve conduction velocities for their sensitivities in detecting differences in neurologic function between patients receiving short dialysis (1 1/2 - 2 hours thrice weekly, and patients receiving long dialysis (3 - 4 hours thrice weekly) therapies. A minimum of 10 patients in each therapeutic group (i.e. 10 on short dialysis and 10 on long dialysis) must be studied completely during this final contract year.

b) Touch pressure and pallesthesia measurements will be compared with clinical symptomatology, physical examination, and nerve conduction velocities for their sensitivities in detecting alterations in neurologic function with correction of the uremic state by transplantation. Renal failure patients will be followed serially with the above mentioned tests throughout their pre-transplantation and post transplantation courses.

Contractor: The Memorial Hospital, Pawtucket, Rhode Island

Amount: $67,520

Title: Antithrombogenic Surfaces: Platelet-Interface Reactions

Objectives: To obtain a better understanding of the interaction between blood and artificial surfaces.

Major Findings:

1) A crude lipoprotein fraction that inhibits platelet adhesion has been separated from normal serum, and preliminary characterization of this preparation indicates that a low density lipoprotein is the major component.

2) Highly purified preparations of albumin and fibrinogen were characterized by gel electrophoresis, labeled with ^{125}I, and exposed singularly and in combination to Cuprophane and polyvinyl chloride (PVC). Both proteins were shown to bind to these surfaces, and the amount of binding of one protein was influenced both by the amount of that protein present and by the amount of a second protein present.

3) The investigators have confirmed their previous studies in which it was shown that human endothelial cells can be grown upon polystyrene, glass, and aged Cuprophane, but not upon new Cuprophane. They have extended these studies to show that endothelial cells can be grown upon purified polyvinyl chloride of polycarbonate. The investigators have determined the relative strength of adhesion of these cultured endothelial cells to supporting media by subjecting the cultured cells to graduated gravitational forces.

Proposed Course:

1) Studies on inhibition of adhesion of platelets to artificial surfaces. This work will include further efforts to purify and characterize a naturally occurring inhibitor of platelet adhesion. 2) Studies on the interactions of plasma proteins and artificial surfaces. This work will include final development of a radioimmunoassay for the various proteins and use of this system for the above studies. 3) Growth of endothelial cells in hollow fibers and tubing under flow conditions. Analysis of influences such as flow rates and media on adhesion and growth. 4) Standardization of an *in vitro* test system for use in quantitation of platelet and leukocyte adhesion to artificial surfaces under blood flow conditions.

Contractor: Minneapolis Medical Research Foundation

Amount: $72,190

Title: Assessment of High and Low Dialysate Flow Rates in Hemodialysis

Objective: To compare dialysis conducted with dialysant flow rates of 500 ml/min and 200 ml/min.

Major Findings: Prolonged exposure (up to twelve months) of 10 patients to Q_D 200 dialysis has resulted in statistically significant reduction in serum cholesterol and elevations of MNCV. Concurrently, the patients arrived at a new steady-state of acid-base balance reflecting greater respiratory compensation of metabolic acidosis. These changes were assumed collectively to represent the effects of 20% reduction in dialysis acetate load.

Proposed Course: The prolonged cross-over study of Q_D 500 vs Q_D 200 will be concluded this year. Calculations of the actual acetate loads will be determined to examine the theory that the improvements noted with decreased dialysate flow rates were secondary to decreased acetate loads.

Contractor: Minneapolis Medical Research Foundation

Amount: $89,628

Title: Acid-Base Regulation in Hemodialysis

Objective: The investigation will study two aspects of acid-base regulation in hemodialysis: 1) the role of acetate in correcting metabolic acidosis and 2) respiratory and EEG responses to repetitive acute corrections of metabolic acidosis in the chronic hemodialysis patient.

Major Findings: Most of time and effort to date has been spent on refinement of the protocol (i.e. determination of safe acetate loads, determination of dialysance, etc.) and establishment of techniques to measure the many dependent variables to be examined. The initial values on acetate kinetics do not corroborate data, from other investigators, which indicate a build-up of blood acetate in all patients with standard acetate loads.

Proposed Course: The experimental design calls for administering a spectrum of acetate loads via dialysis and examining the following dependent variables: 1) acid base compensation; 2) alterations in intermediary metabolism bi-products; 3) blood lipid patterns and 4) neurobehavioral states as determined by MNCV and EEG power spectral analysis.

Initially the acetate loads were to be 0, 100, 300, and 500 mEq/hr. Preliminary work under this contract and the low flow contract indicated, however, that acetate loads greater than 300 mEq/hr resulted in marked alkalosis and might be hazardous to the subjects. Consequently, the current protocol contains acetate levels of 0, 100, 165 (baseline of 35 mEq/L dialysate), 200 and 250 mEq/hr.

Contractor: University of Minnesota

Amount: $78,193

Title: Fluid Dynamics of Blood Cells and Applications to Hemodialysis

Objectives: The contractor has focused his research in two areas:
(1) Development of a novel blood access
(2) Deposition of formed elements onto filterin surfaces

Major Findings: Several modifications of the blood access device has been tested in animals with limited but encouraging success. Extensive experimental tests have been conducted on the deposition of blood elements onto filtering surfaces.

Proposed Course: Work will continue in these two aress of research.

Contractor: University of Missouri

Amount: $20,905

Title: Anti-Nuclear Antibodies in Chronic Dialysis and Renal Transplantation

Objectives: To determine the incidence and significance of anti-nuclear anti-bodies in chronic renal disease patients

Major Findings: Because of the presence of free cell nuclei and nuclear remnants adherent to used dialysis membranes, we are studying the incidence of antibodies to nuclear antigens (DNA, ENA and FANA) in patients with end stage kidney disease, in patients on chronic dialysis, and in patients after kidney transplantation. The incidence of positive patients to date is as follows: end stage kidney disease (pre-dialysis) 7/30 (23.3%), dialysis one to four years 30/116 (25.9%), successful kidney transplantation 5/19 (26.3%), transplantation failure 8.17 (47.0%). No major blood group characteristics have been identified to distinguish the positive group; possible unique HLA-antigen trends are suggested. Patients positive for antibodies have been found in multiple age groups with many types of kidney diseases. There appears to be no difference in the incidence of antibodies in patients who reuse compared to those who do not reuse dialyzers. Rapid changes between antibody positivity and negativity in serial studies suggest the possibility of circulating immune complexes.

Proposed Course: 1) Studies designed to elucidate the significance of antibodies to nuclear material in chronic dialysis patients. The investigators should examine chronic dialysis patients with positive antibody titers for common complications of renal disease. In addition, the researchers should examine chronic dialysis patients with complications of renal disease for increased incidence of positive antibody titers. 2) Studies designed to elucidate the significance of antibodies to nuclear material in chronic renal failure patients prior to dialysis. The investigators should examine chronic renal failure patients with positive antibody titers prior to onset of dialysis for common complications of renal disease. In addition, the researchers should examine chronic renal failure patients with rapid progression of complication of renal disease for increased incidence of positive antibody titers. 3) Studies designed to study the effects of nuclear antibodies on the outcome of renal transplantation and the effect of transplantation on nuclear antibody titers.

Contractor: University of Missouri

Amount: $46,330

Title: Effects of Intra-peritoneal Vasodilators on Peritoneal Clearances

Objective: The objective of these studies will be an attempt to see if peritoneal clearances can be increased by the addition of vasodilators to peritoneal dialysis solutions. Efforts will be directed to see if this can be accomplished with local intraperitoneal effects of the drugs, and minimal or no systemic effects.

Major Findings: To date, the greatest absolute increases in C urea in individual patients are seen with nitroprusside (as much as 5.0 ml/min and 25% of pre mean value). Mean C urea increased in 8 of 8 studies with nitroprusside. The clearance changes shown were not explained by variable drainage volumes; they were the result of significant changes in dialysate/plasma concentration ratios.

Proposed Course: The investigators will continue to examine whether isoproterenol (I), phentolamine (P), hydralazine (H), diazoxide (D), or nitroprusside (N), added to dialysis solutions significantly increase peritoneal clearances of solutes over a spectra of molecular weight from urea (60) to inulin (5000). Secondly, they will see if any effects can be maintained over repeated exchanges and from dialysis to dialysis. Thirdly, in some patients, they will compare the effects of the above mentioned drugs on peritoneal clearances, when administered intravenously, to the effects when administered intraperitoneally. Fourthly, they will determine the amount of these drugs absorbed from the peritoneal exchanges, and blood levels achieved subsequent to intraperitoneal and intravenous administration.

CONTRACTOR: University of Naples

AMOUNT: $23,400

TITLE: Studies on Peritoneal Dialysis

OBJECTIVE: To continue a study which involves new approaches which promise to increase significantly the efficacy of peritoneal dialysis through the novel use of antidiuretic and vasodilating drugs in the peritoneal lavage solution, or of a combination of peritoneal lavage and the use of oral diuretics in the gut. Also to investigate whether there is a relationship between the amount of protein lost into the peritoneal dialysate and the timing of the dialysis since there are indications that the protein leakage is greatest postprandially.

MAJOR FINDINGS: Dr. Giordano has demonstrated during the previous year of his NIH-supported research that inclusion of certain drugs in the peritoneal lavage solution significantly increases the transport of substances which must be removed out of the uremic patient through the peritoneal membrane. This development, in turn, promises to shorten significantly the time needed for peritoneal dialysis treatment for the maintenance of life in uremic patients. In addition, the Principal Investigator has demonstrated the feasibility of brief daily peritoneal dialysis, self-administered, in association with a high calorie-low protein (0.6 gm per kg) diet.

PROPOSED COURSE: The project has two parts. The objective of the first part is to quantify protein losses into the peritoneal dialysate in patients who will be dialyzed after six hours of fasting or after a proteinaceous meal. The second portion of the work will investigate the effect of four vasocactive drugs on peritoneal clearance of sodium if the drug is added in small quantities to the peritoneal dialysate.

CONTRACTOR: University of Naples

AMOUNT: $32,000

TITLE: Studies on Oxystarch in the Treatment of Uremia

OBJECTIVE: 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 and related substances as a sorbent of uremic wastes to regenerate and recycle artificial kidney dialysate solutions.

MAJOR FINDINGS: Oxycellulose, a new polyaldehyde, has been obtained by slow oxidation of cellulose. Oxycellulose has a greater affinity than oxystarch to ammonia, and a somewhat reduced urea-binding activity. The major characteristic of oxycellulose is its insolubility, a property which makes it suitable for dialysis fluid regeneration. In vivo studies of oxycellulose showed no detectable toxicity in mice who received 20% of their diet in the form of oxycellulose, or in in vivo studies in patients. When used as an intestinal urea sorbent, oxycellulose causes a significant decrease in the abnormally elevated BUN of these uremic patients and a corresponding increase in fecal nitrogen.

PROPOSED COURSE: In a long-term study oxystarch will be given to patients by mouth in the form of oxystarch-containing foods. The potential of this treatment for savings in dialysis time will be studied, and if successful, a possible application to patients equipped with indwelling peritoneal catheters is envisioned; the oxycellulose or oxystarch with charcoal will be utilized to regenerate the peritoneal dialysate.

Contractor: Northwest Kidney Center

Amount: $16,000

Title: A Comparison of Home Hemodialysis and Home Peritoneal Dialysis in Patients Trained at a Community Dialysis Center.

Objectives: This study will compare the overall medical effectiveness of home treatment by peritoneal dialysis with that of home hemodialysis in similar patients.

Major Findings: The back-up dialysis rate for medical reasons in peritoneal dialysis patients is no greater than for hemodialysis patients. The overall mortality for patients on peritoneal dialysis has been approximately half as great again as for patients on hemodialysis; this undoubtedly relates to the patients selected for peritoneal dialysis.

Proposed Course: The study will compare medical effectiveness, incidence of complications, degree of social and vocational rehabilitation, and cost of the two (2) forms of treatment. In addition, special studies will be performed to compare the effects of peritoneal dialysis and hemodialysis on platelet survival and lipid metabolism.

Contractor: Northwestern Univesity

Amount: $63,000

Title: Comparison of Maintenance Peritoneal Dialysis and Maintenance Hemodialysis

Objectives: To compare peritoneal and hemodialysis based in the following parameters:
mortality, Morbidity, biochemical levels, liver function, metabolic bone survey, nerve conduction velocity and rehabilitation.

Major Findings: New Contract

CONTRACTOR: University of Pisa

AMOUNT: $25,095

TITLE: Albumin and Hormone Metabolism in Chronic Uremia and Hemodialysis

OBJECTIVE: To elucidate the turnover and biochemical aberrations of albumin, insulin, and growth hormone metabolism in uremia both on and off dialysis.

MAJOR FINDINGS: Sophisticated determination through a new tracer technique has shown that catabolism of insulin was slowed down considerably in uremic patients -- apparently because normally functioning renal tissue is required for a normal rapid breakdown of insulin in the body. It was also found, however, that the rate of secretion of insulin from the pancreas is reduced in uremia -- apparently because of an as yet unknown toxic suppression of normal insulin synthesis in the pancreas or secretion from the beta cells.

Chronic dialysis of several months' duration distinctly improved the fractional catabolic rate, metabolic clearance and insulin delivery rate which are markedly reduced in uremic patients before dialysis treatment. After dialysis of several months these values approach normal values in a near-asymptotic fashion.

PROPOSED COURSE: Studies of ^{125}I-insulin kinetics in uremic patients submitted to regular dialysis program will be continued; these studies are performed at various intervals from the preceding dialysis. Aim of these studies is to evaluate the relationship between insulin metabolism and the plasma concentration of toxic metabolites.

Also, similar studies will continue for growth hormone using basically the same techniques and a new method developed by the group which has already been used successfully in HGH kinetics feasibility studies. Since observed high human growth hormone levels may possibly be a factor in the hypertriglyceridemia of uremia, the proposed study of human growth hormone levels in relation to the frequency of dialysis may yield information of value concerning enhanced atherosclerosis in uremic patients on dialysis.

Contractor: Research Triangle Institute

Amount: $20,000

Title: National Dialysis Registry

Project Description: The contractor will continue to accumulate data for the National Dialysis Registry on chronic intermittent dialysis patients, and quarterly statistical printouts of the total registry patients to the Project Officer and cooperating centers. They will furnish the same data on patients in VA Centers to the Project Officer.

Data files which link patient or center names with data will be maintained in a manner which insures the maintenance of confidentiality of patient and center data; the manner in which these files are handled have been reviewed and approved.

The National Dialysis Registry has collected data since 1969 from virtually all centers engaged in dialysis in the United States, and prepared from the data statistical analyses for use by the NIAMDD 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. Analyses of death by primary cause and multiple decrement life tables are also now available.

Proposed Course: Current plans are to phase out this contract during this fiscal year after transferring data to the new ESRD Medical Information System to be operated by BQA.

Contractor: Southern Research Institute

Amount: $103,991

Title: Activated Carbon Fibers for Use in Artificial Kidney Devices

Objectives: To develop activated carbon fibers suitable for use in artificial kidney absorption devices, and to develop activated carbon hemoperfusion cartridges.

Major Findings: Fibers with carbon loading of 80% have been prepared in wetspinning facilities and wound into coils. Cartridges containing these coils have been evaluated *in vitro* at Gulf South Research Inatitute to determine the rate and capacity for removal of BUN, creatinine, uric acid, inulin and heparin. Several animal experiments are being conducted prior to human experimentation.

Proposed Course: Pre-clinical experiments with sheep will be completed, followed by human trials.

Contractor: Stanford Research Institute

Amount: $91,000

Title: Artificial Kidney - Biochemical Evaluations

Objectives: 1) Isolation and characterization of "middle molecular weight" peptides from biological fluids of uremic patients; 2) Development of procedures for the rapid estimation of these compounds that can be applied by well equipped clinical laboratories; 3) Examination of possible correlations between the levels of these compounds and the pathophysiologic characteristics of uremia. The tasks are to be performed sequentially and therefore all the contractors efforts during the first 12 months should be focused on the peptides' isolation and identification.

Major Findings: During the first several months of the contract period the investigators have been developing a technique to separate the organic acid conjugates of amino acid chains and the water soluble conjugated amino acids. They have now completed this task and have also separated the latter group into amphoteric plus basic amino acid conjugates and acidic amino acid conjugates. Peptide purification and identification have been pursued further in only the amphoteric amino acid conjugates at this time. This fraction has been subjected to electrophoresis-yielding 3 separate bands. Bands 1 and 3, which are ninhydrin positive, were Dansylated and run on one and two dimension Thin layer chromatography. Bands 1 and 3 of the amphoteric conjugated amino acids showed two peptides each on the one dimensional TLC and 4 and 2 peptides, respectively, on the two dimensional TLC.

Proposed Course: Further purification of these peptides will be performed by repeated, alternating subjection to High Pressure Liquid Chromatography and Thin layer Chromatography. Identification of the peptides, which obviously must await purification, is purely in the planning stages. Techniques being looked into are mass spectrometry; amino acid sequencing by the Edman technique of residue tagging; plasma desorption mass spectrometry.

Contractor: Stanford University

Amount: $175,942

Title: The Role of Acetate in Dialysate for Hemodialysis

Objective: To gain a better understanding of the consequences of using acetate as a buffer in hemodialysis

Major Findings: To date most of the efforts of the group have been directed toward task number one - establishment of appropriate assay systems. The enzymatic assay for acetate, acetoacetate, B-hydroxybutyrate, pyruvate and lactate have all been set-up in the laboratory. Minor alterations in the standard procedures have been made to increase the validity and reliability of the tests. mM concentrations are calculated both via standard curves and measured changes in NAD molar concentrations. Dr. Weiner's group has completed multiple studies on the quantitation of the mass transfer of acetate in isolated hemodialyzers and the parameters which affect that transfer. The data on the Dow-4 and Gambro 13.5 artificial kidneys were presented in a paper delivered at the Clinical Dialysis and Transplant Forum.

Proposed Course:

1. Quantitiation of the mass transfer of acetate with a variety of isolated hemodialyzers in vitro and examination of the influence of various factors such as flow rates, acetate concentrations and PH on this transfer;

2. Quantitation of the mass transfer of acetate during hemodialysis of uremic subjects and identification of the factors which influence this process;

3. Determination of the nature of the anion gap which develops during hemodialysis;

4. Determination of the metabolic fate of ^{14}C acetate by infusion of the isotope during dialysis;

5. Acetate tolerance tests in normal volunteers;

6. A pilot study of the effects of acetate on atherosclerosis in rabbits.

Contractor: Stanford University

Amount: $113,384

Title: Hyperglycemia, Hyperlipidemia, and Insulin Resistance in Chronic Renal Failure and in Relation to Hemodialysis

Objectives: The goal has been to a) examine the frequency and severity of hypertriglyceridemia and hyperinsulinemia in a population of patients with moderate to severe chronic renal failure, and b) to determine whether these abnormalities are readily responsive to dietary manipulation.

Major Findings: Fasting plasma triglycerides were 150 mg% in 7 of twelve subjects on the conventional diet, with a group mean of 256 mg%. Fasting triglycerides fell in all subjects on the low carbohydrate diet, to a mean of 169 mg% (P .01), while glucose, insulin, cholesterol, free fatty acids and growth hormone levels did not change. Post-prandial triglyceride levels were lower at each point in time on the low carbohydrate diet. Total insulin response was also lower for 11 of 12 subjects on the low carbohydrate diet. No correlation was found between creatinine clearance, age or relative weight and fasting triglyceride levels. Triglyceride turnover rate, using ^{3}H-glycerol, was measured on the last day of each dietary study period. Results indicated that a decrease in triglyceride concentration seen on the low carbohydrate diet was associated with a decrease in triglyceride production rate.

Proposed Course: Further work will be performed to assess the following: (1) the effect of the diet on glucose and lipid abnormalities, (2) the effect of hemodialysis on glucose tolerance in uremia (3) circulating causes of insulin resistance (4) tissue sites of insulin resistance, and (5) mechanisms of uremic hypertriglyceridemia.

Contractor: University of Tennessee

Amount: $34,734

Title: Detection of Toxicity of Extracted Constituents from Dialyzers and Components

Objectives: To develop *in vitro* tests to evaluate the toxicity of dialyzers and components.

Major Findings: Several hemodialyzers were tested using a standardized protocol developed during the previous contract year. The results indicated significant differences among the unit tested.

Proposed Course:

- The evaluation of dialyzers will be continued
- The substances leached from the units will be identified and tested for its biological activity.

Contractor: University of Texas at Austin

Amount: $54,000

Title: Physiological Transport Parameters in Patients in Peritoneal and Hemodialysis

Objectives: To develop mathematical models to study hemodialysis and peritoneal dialysis regimes and extraction rates limitations due to body inter-compartmental resistances.

Major Findings: The hemodialysis and peritoneal dialysis models have been successfully developed. A significant amount of clinical data has been obtained for preliminary evaluation of intercompartmental and peritoneal resistances for different solutes. Initial conclusions indicate that body resitances significantly impede the removal of metabolites.

Proposed Course: A larger number of patients will be included in the studies to determine intercompartmental and peritoneal resistances for different solutes using the models already developed.

Contractor: University of Utah

Amount: $30,000

Title: Study of Recirculating Peritoneal Dialysis with a Subcutaneous Access System

Objectives: The purpose of this project is to develop and evaluate clinically a new subcutaneous peritoneal cathether and a recirculating, regenerating peritoneal dialysate system.

Specific objectives are: (1) Development of an improved catheter; (2) Optimization of flow regime for recirculating peritoneal dialysis; (3) Development of a satisfactory sterilization system; and (4) Development of a macromolecular leak marker.

Major Findings: Nine patients with end stage renal disease have been treated with peritoneal dialysis utilizing a subcutaneous peritoneal catheter as the access route and recirculation (RPD) as the dialysis format, over a period extending from one to eleven months. Seven of the nine patients were severe diabetics and the other two had lost blood access routes. There were two deaths, six episodes of peritonitis and four cases of subcutaneous infection. Clearance values were equal to or superior to that of standard intermittent peritoneal dialysis, the dialysis format is automated, and preliminary cost estimates show promise of cost equal to or lower than home hemodialysis costs.

Proposed Course: This project will be continued for another year.

Contractor: Vanderbilt University School of Medicine

Amount: $121,478

Title: Neurobehavioral and Clinical Response to Hemodialysis

Objectives: The aim is to develop objective, quantitative measures of uremic dysfunction of the nervous system in patients with renal failure 1) as indicators of adequacy of dialysis and 2) as tools for investigating the pathogenesis of uremic neurobehavioural impairments.

Major Findings: In prospective experiments initial dialysis formats favoring "middle molecule" transport tend to normalize the EEG power spectrum regardless of constant or differential dialysis. Neither improvement nor deterioration is noted in neurobehavioral test measures during the use of Travenol 1.0m^2 and Travenol 1.6M^2 dialyzers. Peripheral nerve function continues to reveal no systmatic change with renal failure or with maintenance dialysis in this center with the exception of ulnar motor response amplitude. The latter measure declines significantly with time on dialysis, a trend which needs further study. Abnormalities in both the electrophysiologic measures (EEG, visual evoked response) and the cognition-dependent measures (continuous memory test, choice reaction time, continuous performance test) vary directly with the degree of renal failure. As maintenance dialysis is begun and continued, all of these measures appear to improve at different rates to stable levels which seem to be unique to each patient. Results in some of the cognition-dependent measures appear to vary with age and to reveal "learning" or "experience" effect with repetitive test sessions. Conventional amounts of dialysis as measured by urea removal may be greater than required by at least some patients with end stage renal disease for maintenance of normal neurobehavioral function and freedom from clinical uremic symptoms.

Proposed Course: They propose to use neurobehavioral response parameters to determine the effects of, and to optimize the relationships between, quantitatively measured and controlled metabolite generation and various dialysis formats, in patients' with chronic renal failure, in order (1) to optimize dialysis treatment, (2) to provide an objective basis for estimating dialysis cost/patient benefit relationships, and (3) to explore further the pathogenesis of clinically significant dialysis-responsive uremic impairment.

Contractor: University of Washington

Amount: $84,995

Title: Acid - Base Chemistry and Human Bone

Objective: The objectives are to understand better the factors contributing to the abnormal biochemical findings identified in renalosteodystrophy and their interrelationship. It has been postulated that the plasma Mg/Ca ratio is the determinant of bone Mg, and that bone high in Mg is not normal bone. Measurements include quantitative histology, whole bone density, deproteinated mineral density, x-ray diffraction, and thermoluminescence. In rats, investigations include variable dietary Mg/Ca ratios, uremia with and without TPTX, and vit. D deficiency. Human studies include evaluation by serial biopsy of variable dialysate Ca and Mg concentrations, 1,25-dihydroxycholecalciferol, and primary versus secondary hyperparathyroidism.

Major Findings: New gravimetric methods have been developed which permit measurement of whole bone density and mineral density, thus calculation of percent mineral and percent matrix. X-ray diffraction and thermoluminescence have been added to biochemical methods. Experiments in rats evaluating normal and high Ca diets after thyroparathyroidectomy have been completed. Preliminary studies of vit. D deficiency and separate studies of a high Mg diet continue to suggest the importance of the plasma Mg/Ca ratio as determinant of bone Mg, and to consequent resultant biochemical and crystal abnormalities. In humans, analyses of results from dialysis patients on different Ca concentrations, $CaCO_3$ supplementation, and 1,25-DHCC continue to emphasize the importance of prevention of hypocalcemia. Comparison of results of bone biopsies in patients with primary and secondary hyperparathyroidism emphasizes the importance of the plasma Mg/Ca ratio in mineral crystal abnormalities. The first phase of a zero Mg dialysis study in patients with advanced osteodystrophy has been completed.

Proposed Course: The investigator will continue bio-chemical and histologic analyses of current biopsy material. The effects of zero Mg^{++} dialysate on bone mineralization will be further evaluated.

Contractor: University of Washington

Amount: $142,116

Title: Pharmacokinetic Studies in Chronic Renal Disease and Hemodialysis

Objective: This contract, which has been let for an 18 month period, is designed to examine the clearance of some important therapeutic agents in patients with chronic renal failure and dependence on hemodialysis. The medications being studied during the current contract period are: (1) Quinidine; (2) Procainamide and its metabolite N-aetyl-procainamide; (3) Flucytosine; (4) Azathioprine; (5) Clofibrate.

Major Findings: The quinidine studies performed to date have been in vitro determinations of the degree of protein binding. The investigators, using mass spectroscopy to measure quinidine levels, have shown approximately 60% of the drug to be protein bound in vitro. Clinical studies of flucytosine in normal subjects have shown almost complete absorption of the drug when given orally, although the rate of absorption is some what slowed when the agent is given with a meal. Comparison of the area under the serum curve following oral and intravenous administration in the same subject with the urinary excretion of unchanged drug in the urine demonstrated a bioavailability of 80-100% and no evidence of biotransformation. This lack of biotransformation was also demonstrated in six patients with varying degrees of renal insufficiency who received the drug intravenously. Renal and plasma clearances were nearly identical and the non-renal rate of removal in a surgically anephric patient was minimal (1.3 ml/min).

Proposed Course: The procainamide work to date has centered on the synthesis of N-acetylprocainamide and the development of a highly sensitive and specific assay for this metabolite. Once in vitro tests indicate the capacity to accurately measure this metabolite and the parent compound, in vivo studies will be performed on 10 patients by the administration of procainamide. The investigators are also considering seeking clearances for administration of N-a-procainamide. The azathioprine studies are currently being directed toward an assessment of the various metabolites of the parent compound. Once the metabolites are identified, by use of gas chromatography and mass spectroscopy, measurements will be made in patients receiving the drug therapeutically and correlations made between the clearances and level of residual renal function. A potentially significant outgrowth of this work would be a definition of the clinically beneficial metabolites.

Clofibrate analysis is still in the very early stages. Preliminary studies indicate a high level of protein binding, approximately 95%, and very little clearance of parent compound. Work will proceed on the identification of probable metabolites, then on the establishment of an assay for the significant compounds, and finally on to the measurements of the clearances of these substances in renal failure patients.

Contractor: University of Washington

Amount: $70,000

Title: Cannula Research

Objective: To design and test improved vascular access for hemodialysis.

Major Findings: In animal work using single fistula catheters the investigators have shown that the variable of insertion site affects functional longevity; sites right at the fistula being least favorable. Also, catheter functional life was shown to correlate with catheter length. Uncontrolled catheter movement, produced by pulsatile blood flow emerging from A-V fistulas was shown to cause vessel wall damage and thrombosis. Preliminary testing of immobilized catheters, designed to avoid vessel wall damage, has produced encouraging results and tests are continuing.

Single fistula catheters have been implanted in 8 patients with successful function to 19 months. Catheters were used successfully for dialysis. The variable of catheter intravascular tip shape has been identified through these trials as an important factor in long term catheter function. Results show the catheter to be a helpful new method of blood access which could be superior to A-V cannulas and to dialysis by needle puncture.

Proposed Course:

1. As a continuation of prior work, a vascular access device, the single fistula catheter with rounded tip, will be evaluated in dialysis patient trials.

2. In additional work to be conducted in 15 animals, the adequacy of bovine arterial heterografts as substitute vascular channels for the fistula catheter will be assessed.

3. Work will be conducted in 15 animals to evaluate performance of a double-ended immobilized fistula catheter.

   Implant performance in all groups will be evaluated according to criteria of functional longevity, incidence of complications, adequacy of initial site, and cause of failure.

Contractor: University of Washington

Amount: $125,186

Title: Hyperlipidemia and Atherogenesis in Renal Failure

Objectives: To delineate the mechanisms of hyperlipidemia and accelerated atherosclerosis in renal failure patients who are either on hemodialysis or have undergone renal transplantation.

Major Findings: Recently completed studies in uremic animals in the principal investigator's present contract indicate that the adaptation to caloric deprivation may be accelerated in renal failure. In addition, recently completed preliminary studies in man and an experimental animal model suggest that impairment in the removal of triglyceride-rich lipoprotein from plasma rather than increased hepatic production may be the principal mechanism contributing to hyperlipidemia in uremia.

Proposed Course: The following areas will be investigated:

(1) Apolipoproteins in renal failure patients

(2) The effects of serum from acutely and chronically uremic rats on adipose tissue lipoprotein lipase

(3) Insulin-inducible lipogenic enzymes in the liver of chronically uremic rats

(4) Adaptation to fasting in chronic uremia.

Contractor: University of Washington

Amount: $150,250

Title Quantitation of Minimum Adequate Dialysis Requirements

Objectives: The purpose of this research program to investigate the possibility that in the uremic subject there are toxic substances in the molecular weight range 500 to 5,000 daltons. On the basis of past clinical experience, it is possible that a major manifestation of intoxication from these "middle molecular weight substances" (middle molecules - MM) is uremic peripheral neuropathy. This research is designed to test this hypothesis using prospective hemodialysis protocols designed to evoke MM intoxication. In the course of these studies the investigators will look for other manifestations of MM intoxication using carefully selected and developed serially measured patient parameters to monitor patient well-being. In the course of these investigations, they also plan to study metabolism of these substances including generation rate, volume of distribution, renal handling, and extrarenal removal rates.

Major Findings: The investigators have postulated a dialysis index which is a calculated ratio of the predicted removal of a model "middle molecule" compared to an arbitrary standard. Patients are studied prospectively in dialysis therapies designed to have a Dialysis Index (DI) of greater or less than 1. Seven patients have dialyzed for 6 months to 4 years with a DI>1.0. Dialysis time has been between 5.0 and 18 hours per week. None of these patients have developed any evidence of inadequate dialysis, and all feel as well or better than with their previous longer dialysis period. Eleven patients have had their dialysis techniques altered to result in a DI of <1.0. All of these patients had endogenous GFR's of less than 1.0. All remained on this protocol for greater than 6 months with dialysis times between 7.5 and 18 hours per week. Of the 11 patients, 4 showed deterioration in motor nerve conduction velocity (MNCV), one developed pericarditis, and one had a decrease in well-being (significant decrease in CPT scores). Another patient's MNCV appears to be deteriorating but has not yet reached statistically significant levels at this time. This makes the proportion of toxicity between 36% to 64% for the group with a DI<1.0 (depending on how one classifies these clinical results) compared to 0% for those with a DI>1.0. These results suggest that when endogenous (residual GFR) and exogenous (dialysis efficiency) removal of middle molecules is reduced below a DI of 1.0, there is a significant increase in the probability that uremic toxicity will occur or recur.

Proposed Course: This project will be concluded in the coming year.

CONTRACTOR: University of Washington

AMOUNT: $69,928

TITLE: Anticoagulant-Bound Hemodialysis

OBJECTIVES: Animal evaluation of a heparin grafted hollow fiber dialyzer will be completed. Similarly animal tests will be completed on tubing sets and drip chambers which have been treated with ionically bound heparin. Clinical testing of the combined heparin treated system will occur, first with a few exposures per patient and later for a three month trial in two patients.

Animal experiments using a totally heparingrafted dialysis system (HFAK Cordis-Dow Model 4) will be phased out after 15 experiments, designed to decrease the variations obtained so far with Model 4.

Permeability studies of HFAK Model 4 will be carried out in vitro in 5 dialyzers before and after heparingrafting. Permeability studies include urea, creatinine and B_{12}. Furthermore, possible decrease of tensile strength after heparingrafting will be checked.

Assuming favorable permeability parameters clinical trials will start in chronic uremic volunteers with a totally heparingrafted dialysis system (HFAK Cordis-Dow Model 4). A total of 100 anticoagulant bound hemodialyses are planned sequentially in chronic uremic volunteers. A complete coagulation workup including quantitative fibrinogen and a complete hematological workup (including free plasma hemoglobin) is planned before, at the end and in the middle of dialysis, identical to the data collected in sheep. Chemical data will include urea and creatinine levels before and after dialysis and loss of fluid.

Antithrombogenicity of the system will be assessed by Δp, fiber loss %/hour and macroscopic inspection.

MAJOR FINDINGS: Using the cyanogen bromide method, heparin has been bound covalently to the Hollow Fiber Artificial Kidney (HFAK) Cordis-Dow Model III, retaining biological activity as an anticoagulant. Three different reaction conditions for the binding heparin to the hollow fibers (HF) have been applied and subjected to in vivo test. In vivo testing of these three series in sheep using a carotid-jugular double shunt system revealed increased antithrombogenicity of the heparin-bound HFAK. While blood flowrates in heparin-grafted HFAKs series A, B and C remained fairly stable at about 200 ml/min throughout the experiments (7 hours), a progressive decrease of blood flowrate to less than 100 ml/min within 5 hours was found in non-grafted dialyzers. In vitro clearance in heaprin-grafted kidneys showed a decrease of creatinine clearance after grafting from 85 ml/min to 77 ml (minus 10%) but an appreciable decrease of vitamin B_{12} clearance from 12.0 ml to 7.0 ml/min. This reduction in membrane permeability has to be minimized to use the heparin grafted HFAK in the treatment of chronic uremia. A method was developed for heparin treating blood tubing sets by use of a cetylpyridinium chloride complex.

Proposed Course: In this contract year the clinical utility of the presently developed anticoagulant bound system is expected to be demonstrated. The project may be continued to further test any new developments in methodology.

Contractor: Yale University

Amount: $105,287

Title: Carbohydrate Intolerance in Uremia: Insulin Resistance, Hyperglucagonemia and Altered Protein Metabolism

Objective: The objectives of this study are as follows: (1) to quantitate the degree of overall tissue resistance to insulin in uremia; (2) to define the contribution of increased hepatic glucose release to insulin resistance; (3) to assess the role of hyperglucagonemia and altered tissue responsiveness to glucagon in any observed abnormalities in hepatic glucose balance; (4) to evaluate the amino acid response to protein ingestion in uremia and compare it with that observed in diabetes mellitus.

Major Findings: Hyperglucagonemia in uremia is a result of decreased catabolism rather than hypersecretion of this hormone. Sensitivity to the hyperglycemic effects of glucagon is increased in undialyzed uremic patients. Dialysis normalizes the glycemic response to glucagon, possibly accounting thereby for improved glucose tolerance despite persistent hyperglucagonemia. These data suggest that glucose intolerance in uremia depends on the interplay between circulating levels and tissue responsiveness to glucagon. Furthermore these findings provide the first evidence of decreased hormonal catabolism contributing to a hyperglucagonemic state and of altered tissue sensitivity contributing to the pathophysiologic action of this hormone.

Proposed Course: The proposed work will involve two areas of study: (a) "glucose clamp" studies to quantify and characterize insulin resistance in uremia; (b) glucagon-binding studies in experimental uremia to evaluate the increased sensitivity to glucagon observed in uremic patients.

ANNUAL REPORT SUMMARY
EPIDEMIOLOGY AND FIELD STUDIES BRANCH

Southwestern Field Studies Section

Studies relating primarily to the epidemiology of diabetes mellitus and its vascular complications amongst the Pima Indians have been continued. The biennial examination of each member of the Gila River Indian Community population who volunteers for diabetes and arthritis related studies provides basic data from which factors which precipitate diabetes and which relate to the development of the vascular complications of diabetes are evaluated. More intensive investigations on selected subgroups of the community have also been pursued.

Evidence of hyperinsulinemia as a characteristic of both normal and prediabetic Pima Indians has been extended. Evidence that the age of onset of the hyperinsulinemia occurs in the teen-age years has been obtained and that insulin levels in the nondiabetic population beyond this age are two to three fold greater than those of Caucasians has been confirmed. As a result of these findings, investigations have now been initiated to determine the characteristics of insulin receptors in normal, prediabetic, diabetic Pima Indians, and Caucasians.

Studies have been initiated which will enable the investigation of possible intracellular metabolic defects in isolated fibroblast cell cultures among the Pima. It is hoped that both of these approaches will allow understanding of the mechanisms of hyperinsulinemia and of its possible relationship to the propensity of the Pima Indians to develop diabetes mellitus.

Evidence of alternative hypotheses concerning the factors relating to the development of diabetes in the Pimas have been pursued. Extensive studies of glucagon suppression and stimulation (by arginine) have been conducted in prediabetic and normal Indians and Caucasians. No evidence that any abnormality of glucagon secretion is present in the prediabetic state has been obtained. However, evidence that an alpha cell abnormality is present in diabetes of recent onset in the Pimas which is, at least in part, independent of beta cell function has been obtained. Glucagon suppression mediated by the administration of intravenous glucose also appears to be independent of the beta cell abnormality since in recent onset diabetics with no evidence of insulin secretion in response to a glucose infusion, suppression was noted although the degree of suppression was somewhat reduced compared to that of normal Indian and Caucasian controls. Thus, evidence that an alpha cell abnormality may occur early in the course of diabetes in the Pima Indian has been obtained.

Studies of muscle capillary basement membrane thickness in Pima Indian diabetics, prediabetics, normal Indians and Caucasian controls have indicated that muscle capillary basement membrane thickening is characteristic of Pima Indians with diabetes and that the absolute thickness of the capillary basement membrane is strongly related to the duration of known glucose intolerance. Moreover, sensitivity and specificity of the methods of basement membrane measurement have been compared in collaboration with Dr. Siperstein and Dr. Williamson and evidence that the osmic acid fixation technique is rather more sensitivity for the detection of basement membrane thickening in the diabetic of recent onset (and the prediabetic) has been obtained. Future studies involving the follow up of individuals already measured will eventually enable a determination of the prognostic significance of muscle capillary basement membrane thickening to be made.

Understanding of the relationship between obesity and glucose tolerance is important if the mechanisms by which obesity leads to an increased incidence of diabetes are to be understood. The relationships have been explored in the Pima Indian population data and it has been shown that obesity in the absence of discrete evidence of glucose intolerance is associated with increases in the mean glucose level following a carbohydrate load and that the definition of diabetes among the obese requires some modification of the criteria for diagnosis in order to minimize misclassification of diabetic and nondiabetic subjects. Surprisingly the proportion of Pima Indians falling in the hyperglycemic component of the previously described bimodal frequency distribution curves of glucose tolerance is similar in decades beyond 35 years of age, whereas between 25 and 34 years, the decade in which the incidence of diabetes appears to be greatest, the proportion of subjects lying in the hyperglycemic component of the frequency distributions is increased. The observations are consistent with the hypothesis that significant deterioration of glucose intolerance is associated with a relatively temporary weight gain. These relationships will be studied using the now available 10 year diabetes incidence data in the Gila River Indian population.

The incidence of diabetes in relationship to the degree of obesity level of glucose tolerance and the family history of diabetes has been further evaluated. The risk of developing diabetes is much greater among the offspring of two diabetic parents than among the offspring of nondiabetic parents. Factors other than the genetic makeup play an important role in this determination. Obesity, for example, is more frequent among the younger offspring of diabetic parents than in those of nondiabetic parents, suggesting that obesity may be a reflection of the genetic makeup of the diabetic genotype as well as a factor closely associated with the precipitation of glucose intolerance.

Analysis of the data concerning the vascular complications of diabetes amongst the Gila River Indian Community has also been pursued. Evidence that retinopathy is primarily a characteristic of those with two hour post load plasma glucose levels equal or greater than 200 mg/dl has been obtained and evidence that the development of retinopathy is closely related to the duration of known carbohydrate intolerance has now been published. Further investigation of risk factors relating to retinopathy has been undertaken to determine what specific factors relate to the development of retinopathy in the longitudinal data now available over a 10 year period on a significant proportion of the Pima Indian population. Evidence that coronary heart disease is a much less frequent complication of diabetes amongst the Indian population has been obtained and factors relating to its occurrence have been investigated. While the Pima Indians with diabetes develop coronary heart disease more frequently than those without diabetes, it is apparent that even the Pima Indian diabetic has no greater chance of developing coronary heart disease than his nondiabetic Caucasian counterpart. The risk factors relating to this apparent protection include lower serum cholesterol levels and less frequent cigarette smoking than Caucasians. In this connection it is of interest that the Pima appear to have much higher ratios of HDL to LDL cholesterol than do Caucasians, suggesting perhaps that the high level of HDL cholesterol may have a protective effect. Investigations of HDL cholesterol levels in Indians and Caucasian diabetics and nondiabetics with and without coronary heart disease will be performed to elucidate further the possible significance of this hypothesis.

Arthritis related investigations during the past year have concentrated primarily on the relationship between HLA B27 and spondylitis. The association of HLA B27 and ankylosing spondylitis among the Pima is less strong than in Caucasian populations. Furthermore, we have found that the association between spondylitis and HLA B27 among the Pima Indians is limited to the male population and that females with sacroiliitis have a similar proportion with B27 positivity as in the population in general. Family studies of the joint aggregation of HLA B27 and sacroiliitis, the radiologic marker for ankylosing spondylitis have been performed, but because of the high frequency of HLA B27 and sacroiliitis in the population little evidence of familial aggregation of the two characteristics could be discerned. It is planned to attempt to seek alternative lymphocyte markers associated with the occurrence of spondylitis in the Pima Indian population using techniques of mixed lymphocyte culture in the hope that a more specific antigen than HLA B27 can be identified.

METABOLIC DISEASE EPIDEMIOLOGY UNIT

Evidence of the antithyroid activity of Escherichia coli as a goitrogen led to the development of a test for the presence of antibody to the goitrogen by hemagglutination techniques. These techniques have been applied to determine the prevalence of antibody occurrence in the population. The relationship of the presence of these antibodies to the occurrence of goiter will be determined.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69,000-11 EFSB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Diabetes Mellitus in the Gila River Indian Community

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

PI:	P.H. Bennett, Chief	EFSB, NIAMDD
OTHER:	P.J. Savage, Staff Physician	SFSS, NIAMDD
	S.L. Aronoff, Staff Physician	SFSS, NIAMDD
	W.C. Knowler, Staff Associate	SFSS, NIAMDD
	M. Miller, Case Western Reserve University	
	E.J. Ballintine	NEI

COOPERATING UNITS (if any)

Indian Health Service
Case Western Reserve University
National Eye Institute

LAB/BRANCH

Epidemiology and Field Studies Branch

SECTION

Southwestern Field Studies Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.9	1.6	5.3

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

The natural history of the various manifestations and complications of diabetes mellitus will be studied and evaluated in an Amerindian community. Various associated conditions and factors, including heredity, that may have a bearing on the frequency and severity of diabetes and/or its complications will also be investigated and selected findings will be contrasted with similar studies performed on other populations with different characteristics or environment.

This will be done in connection with a prospective study of arthritis and rheumatism on the Amerindian population of the Gila River Indian Reservation. Examination will be carried out in a portable clinic building adjacent to the outpatient wing of the PHS Indian Hospital at Sacaton, Arizona, and will consist of a clinical interview, physical examination and special examinations to detect chronic vascular changes (soft tissue radiographs, photographs of the ocular fundi, electrocardiograms, etc.). In addition, glucose tolerance tests and other biochemical and serological tests will be performed.

PHS-6040
(12-75)

Project Description

Objectives:

1. Determine the prevalence and incidence of 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 subclinical 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 Employed:

1. The following tests and examinations are 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 and in addition on those aged 15 years and over
   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. Incidence of diabetes: Among subjects one-half or more Pima and who, at their first examination, were 15 years or older, resident on reservation, and had a two hour plasma glucose less than 200 mg/dl and were not on drug treatment for diabetes, the crude 8 year cummulative incidence of diabetes (defined as 2 hour plasma glucose of 250 mg/dl or greater or history of drug treatment for diabetes) was found to be 23.7%. Factors associated with increased risk included younger age, higher initial two hour plasma glucose, higher body weight index, and diabetes in parents. Preliminary estimates have been made of age-specific incidence density rates of diabetes, and investigations will be made of potential selection biases which could distort the observed incidence rates.

2. Retinopathy and diabetes: The prevalence of retinal changes and their relationship to glucose tolerance, duration of diabetes and age in the

Pima Indian population was determined. Among 1640 subjects age 15 and over retinal changes were found nine times as often in subjects with two hour post load plasma glucose levels >200 mg/dl than in those with lower levels. Some 50% of those with diabetes of 10 or more years duration had retinal changes compared to 11% among those with duration of diabetes of up to 5 years. No important relationship was found with age after stratification for duration of diabetes and no difference in the occurrence of retinopathy between males and females was found. These findings have been prepared for publication.

3. Obesity and Hyperglycemia: The parameters of the distributions of glucose tolerance among 3000 Pima Indians were examined according to age, sex and degree of obesity. As previously reported such distributions are bimodal. The presence of obesity has little effect on the mean glucose level or the proportion of subjects lying in the hyperglycemic component of the distribution except in the 25-34 year old group where the incidence of diabetes is greatest, and the proportion of hyperglycemics among the obese was significantly greater than among the less obese. The mean levels of the first component of the distribution, however, were consistently greater among the obese and this change accounted for the higher overall mean glucose levels among the obese in most age groups. Among the obese it is necessary to use higher glucose values to minimize the misclassification of subjects as "normal" or "hyperglycemic." The results will be utilized to determine the incidence of diabetes and its relationship to obesity in longitudinal studies.

4. Coronary Heart Disease and Diabetes: The frequency of electrocardiographic evidence of coronary heart disease (CHD) and the rate of autopsy proven myocardial infarction were determined in the Gila River Indian Community. The electrocardiograms of 701 Pimas, aged 40 years and over, 45% of whom had diabetes, were read according to the Minnesota Code, and 120 post mortem examinations were reveiwed for evidence of myocardial infarction.

   The frequency of CHD as evidenced by major Q wave changes in the Pima (1.6%) was about one-half that found in Tecumseh, Michigan. The subjects with diabetes had a higher rate of CHD than nondiabetics, both electrocardiographically and at post mortem examination, although the differences were not statistically significant. The low prevalence of CHD in the living Pima and the low rate of infarction at autopsy indicate a low frequency of CHD despite the extraordinarily high prevalence of diabetes mellitus. These findings, which have been prepared for publication, suggest that risk factors other than diabetes mellitus <u>per se</u> play a major role in determining the frequency of coronary heart disease as a complication and often as a cause of death in patients with diabetes mellitus.

5. HLA Typing and Diabetes: HLA typing data has been accumulated on more than 350 subjects, approximately half of whom have diabetes mellitus and the remainder who are >55 years of age and do not have diabetes (controls). The association of HLA-B8 and diabetes in insulin-requiring Pima Indian diabetics could not be confirmed and infact only a single HLA-B8 type in a full-blood American Indian has been found, indicating the rarity of such a type in American Indians.

   Further typing is presently being undertaken among Pima Indian diabetics of juvenile onset and their available first degree relatives. It is hoped that the failure to detect HLA-B8 associated disease may lead to the detection by MLC typing of alternative (and perhaps more speicific) markers associated with diabetes mellitus.

6. Hyperinsulinism and Its Age of Onset in Pima Indian Children: The finding of elevated insulin levels in Pima adults compared with glucose and weight matched adult Caucasian controls led to investigation of whether hyperinsulinism was present in Pima children. Insulin levels were measured during a 75 gm oral GTT in 87 Pimas with normal glucose tolerance aged 5-24 years and compared to levels in 132 5-24 year old Aleuts. Fasting insulin levels in 5-9 year old Pimas were 9.5±1.7 μU/ml. Fasting levels in 10-14 year old Pimas (22.4±1.8 μU/ml) were significantly greater ($p<0.01$) than fasting levels in 10-14 year old Aleuts (6.7± 0.4 μU/ml) and 5-9 year old Pimas. Similar differences (Pima 20.4±2.4 μU/ml-Aleut 8.5±1.3 μU/ml) ($p<0.01$) were found in 15-24 year old subjects. These differences persisted despite adjustment fow weight. The pattern in two hour insulin levels was similar to that seen in fasting levels.

   Insulin levels in Pima children appear to double between the ages of 5-9 and 10 years and over. The hyperinsulinemia observed in Pima adults does not appear to be present from birth but to develop after the first decade of life. This hyperinsulinemia may be related to the Pimas predisposition to develop both obesity and diabetes.

7. Cholesterol and Triglyceride Concentrations in Diabetic and Nondiabetic Pimas: Abnormalities in lipid levels are well-known in diabetics. We have obtained fasting samples in 700 Pimas aged 5-85 with glucose tolerance ranging from normal to markedly abnormal in an attempt to correlate levels of cholesterol and triglyceride with age, glucose tolerance and insulin levels. Preliminary analysis indicates that cholesterol levels are slightly higher in diabetic than in age-matched nondiabetic subjects. Triglyceride levels are elevated in diabetics and correlate with the degree of hyperglycemia. More detailed analysis of these relationships and analysis of their relation to insulin levels is underway.

8. Incidence of Complications of Diabetes: Studies of renal and retinal complications of diabetes and of mortality are being conducted in order to estimate the effects of potentially treatable risk factors, such as hypertension and obesity, on the incidence of these complications. The analyses make use of multivariate techniques for controlling confounding factors.

Significance to Biomedical Research and the Program of the Institute: Since the onset of diabetes mellitus is often insidious, and the disease may remain asymptomatic, it is not possible in most situations to determine the incidence (rate of occurrence of new cases) of diabetes. Estimates of prevalence (fraction of people with the disease) are more readily available. This longitudinal study of diabetes in the Gila River Indian Community evaluates both well and diseased subjects, yielding objective measures of disease occurrence, including incidence rates of diabetes and of its complications. There are some advantages in using incidence rather than prevalence rates in research into causal relationships. Conditions at the time of the development of a disease are closer in time to the cause(s) than are conditions at a later time that might be observed in a prevalence study. Incidence rate directly reflectsthe occurrence of illness, whereas prevalence rates depend also on disease duration (or patient survival). Thus, as the length of follow-up in the study is becoming sufficient, an emphasis has been put on estimating incidence rates. The incidence of diabetes has been shown to depend on family history, age, body weight, and prior level of "normal" plasma glucose. Hyperglycemia was shown to be a risk factor for retinal changes in diabetes, with longer durations of hyperglycemia conferring greater risk of abnormality. Further investigations may delineate additional risk factor (including potentially treatable conditions) for the development of complications in diabetics.

The prevalence of coronary heart disease was low, despite the high prevalence of diabetes, suggesting that there are other important risk factors for coronary heart disease in diabetics besides diabetes per se. Obesity was shown to affect the plasma glucose levels of nondiabetics, which may have implications in selecting diagnostic criteria for diabetes. Preliminary evidence indicates that the HLA-B8 type is not a cause of diabetes in Pimas (as has been suggested in other populations). This finding may stimulate the search for other genetic markers or environmental causes of diabetes mellitus. The finding that hyperinsulinism appears to develop over the age of 10 suggests that the Pimas may develop insulin resistance during childhood. It is hoped that further studies in this area may shed light on the pathogenesis of diabetes in the Pima Indians.

Proposed Course: The long-term prospective studies of diabetes and its vascular complications will be continued. Since many subjects have now had documented glucose intolerance for eleven years, detailed analysis of the factors related to the incidence, rather than prevalence, of the specific complications will be initiated. Such analyses offer a much greater likelihood for identification of factors of causal significance than is possible with prevalence data alone.

Further studies to characterize factors related to the onset of glucose intolerance will also be undertaken and will include determination of the relationship of insulin secretion and obesity to the evaluation of glucose intolerance.

Publications:

1. Bennett, P.H., Rushforth, N.B., Miller, M., and LeCompte, P.M.: Epidemiological Studies of Diabetes in the Pima Indians. Recent Progress in Hormone Research 30:333-376. (in press)

2. Bennett, P.H.: Deteccion, Diagnostico y Registro de Diabetes Mellitus. Organizacion Panamerican de la Salud, Oficina Sanitaria Panamericana, Grupo de Estudio Sobre Diabetes Mellitus, Publication Cientifica No. 312, 1975, p. 34-40.

3. Bennett, P.H. and Miller, M.: Vascular Complications of Diabetes in American Indians, Japanese, and Caucasians: Proceedings, II. Symposium on Diabetes Mellitus in Asia, Kyoto, 1975. Excerpta Medica, Amsterdam. (in press)

4. Hamman, R.F., Bennett, P.H., and Miller, M.: The Effect of Menopause on Serum Cholesterol in American (Pima) Indian Women. Am. J. Epidemiol. 102:164-169, 1975.

5. Rushforth, N.B., Bennett, P.H., Steinberg, A.G., and Miller, M.: Comparison of the Value of the Two- and One-hour Glucose Levels of the Oral GTT in the Diagnosis of Diabetes in Pima Indians. Diabetes 24:438-546, 1975.

6. Dorf, A., Ballintine, E.J., Bennett, P.H., and Miller, M.: Retinopathy in Pima Indians: Relationship to Glucose Level, Duration of Diabetes, Age at Diagnosis of Diabetes, and Age at Examination in a Population with a High Prevalence of Diabetes Mellitus. Diabetes (in press)

7. Ingelfinger, J.A., Bennett, P.H., Liebow, I.M., and Miller, M.: Coronary Heart Disease in the Pima Indians: Electrocardiographic Findings and Postmortem Evidence of Myocardial Infarction in a Population with a High Prevalence of Diabetes. Diabetes (in press)

8. Savage, P.J., Hamman, R.F., Bartha, G., Dippe, S.E., Miller, M., and Bennett, P.H.: Serum Cholesterol Levels in American (Pima) Indian Children and Adolescents. Pediatrics (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 69,001-07 EFSB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Complications and Outcome of Diabetic and Prediabetic Pregnancies

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

PI:	P.H. Bennett, Chief	EFSB, NIAMDD
OTHER:	W.C. Knowler, Staff Associate	SFSS, NIAMDD
	S.L. Aronoff, Staff Physician	SFSS, NIAMDD
	P.J. Savage, Staff Physician	SFSS, NIAMDD

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Epidemiology and Field Studies Branch

SECTION

Southwestern Field Studies Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	0.3

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

To determine the effects of diabetes mellitus and prediabetes on the complications and outcome of pregnancy in women in the Gila River Indian Community.

The characteristics of all pregnancies among residents of the Gila River Indian Community will be determined by review of the hospital records over a ten year period. Each pregnant woman will have a modified glucose tolerance test during the third trimester and following delivery and will be retested at approximately two yearly intervals in conjunction with the prospective study of diabetes mellitus in the same community. The characteristics of the pregnancies in women who subsequently become diabetic will be compared to those among women who were diabetic at the time of pregnancy and those who remain nondiabetic.

PHS-6040
(12-75)

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 Employed: The course and outcome of pregnancies in all female residents of the Gila River Indian Community (Sacaton Service Unit area) are followed and recorded in a standardized manner.

All patients in the prenatal clinic of the Sacaton Hospital receive a modified glucose tolerance test during the third trimester of their pregnancy. These patients receive another modified glucose tolerance test 24-48 hours post partum and are retested at approximately two yearly intervals to document the development or lack of development of diabetes. Infants will be examined to determine the presence of congenital anomalies and other characteristics, and data related to mortality and morbidity will be compiled. At age five years the children will be reexamined to detect any further abnormalities which may have been inapparent at birth.

Major Findings: Analysis has been deferred pending collection of additional data.

Significance to Biomedical Research and the Program of the Institute: In view of the high prevalence of diabetes in the Pima Indians, studies in the Gila River Community provide a unique opportunity to evaluate the outcome of both prediabetic pregnancies and pregnancies with mild carbohydrate intolerance. Retrospective studies of the course of prediabetic pregnancies have to date produced conflicting results. Although it is well established that patients with severe glucose intolerance, especially those with diabetes of long duration and significant microvascular complications, have an increased prevalence of fetal mortality and maternal morbidity, it is not certain whether treatment will improve the outcome of those pregnancies in which there is mild glucose intolerance. Limited prospective studies of the benefit of treating pregnant women with mild glucose intolerance have so far yielded conflicting results. With the recent adoption by the Indian Health Service of more stringent criteria for the treatment of mild carbohydrate intolerance in pregnancy it is hoped that this study will be able to add to the evaluation of this problem by monitoring the impact of these therapeutic changes on the outcome of pregnancies on the reservation.

Proposed Course: The pregnancy study will continue in conjunction with the study of 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.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69,003-03 EFSB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Evaluation of the Prediabetic and Diabetic State in the Pima Indians

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

PI: P.H. Bennett, Chief — EFSB, NIAMDD

OTHER: S.L. Aronoff, Staff Physician — SFSS, NIAMDD

P. Gorden, Clinical Director — NIAMDD

R.H. Unger, University of Texas Southwestern Medical School at Dallas

M.D. Siperstein, University of California Medical School at San Francisco

J.R. Williamson, Washington University School of Medicine

COOPERATING UNITS (if any)

University of Texas at Dallas
University of California at San Francisco
Washington University

LAB/BRANCH

Epidemiology and Field Studies Branch

SECTION

Southwestern Field Studies Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

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

Age, sex, and weight matched groups of genetically normal and prediabetic subjects were examined by means of oral and intravenous glucose tolerance tests, and arginine infusion test with glucose, insulin and glucagon determinations, and measurement of the thickness of the muscle capillary basement membrane to determine whether or not abnormalities in any of these measurements are characteristic of the prediabetic state.

PHS-6040
(12-75)

Project Description:

Objectives: The natural history of diabetes mellitus has yet to be well elucidated. A prospective study of the genetically suspect prediabetic subject (defined as the nondiabetic offspring of two diabetic parents) appears to be the most direct and reliable method of describing the natural history of the disease.

Study of the prediabetic will enable us to better understand the interrelationship of hyperglycemia and microangiopathy which is presently highly controversial and poorly defined. This is possible through the study of chemical and physical abnormalities in prediabetics. Chemical abnoramlities may include subtle changes in glucose and insulin as well as possible specific urinary protein abnormalities. The major physical abnormality under investigation is the muscle capillary basement membrane thickness.

Possible capillary basement membrane thickening, prior to glucose intolerance has been reported by Siperstein, but challenged by Williamson. Variation in findings in part are due to variation in techniques. Resolution of the conflict is of utmost importance in understanding the interrelationship of glucose tolerance and microangiopathy.

The purposes of the study are:

1. To study whether or not carbohydrate abnormalities, insulin abnormalities, capillary basement membrane hypertrophy and urinary protein abnormalities are characteristic of prediabetes.

2. To compare the two major techniques of basement membrane measurement.

3. To initiate a long-term prospective study of prediabetes.

Methods Employed: Two groups were selected for study - the nondiabetic offspring of two diabetic parents and the nondiabetic offspring of two normal parents.

The two groups were matched for age, sex and percent desirable weight and following a dietary preparation were admitted to the Clinical Research Center for testing. Tests included an oral glucose tolerance test, intravenous glucose tolerance test, cortisone glucose tolerance test, an arginine infusion, a quadriceps needle muscle biopsy, fundus photographs and 24-hour urine collection for determination of specific urinary proteins by immunodiffusion.

Results:

1. Muscle Capillary Basement Membrane Thickness (MCBMT): Results are presently available on 17 of 35 prediabetic and 18 of 26 normal Pima Indians.

By the Williamson method the mean MCBMT in the normal (858±48 Å) and prediabetic (887±71 Å) Indians were similar. The mean MCBMT by the Siperstein method was 1466±46 Å in the normal Indians and 1646±105 Å in the prediabetics, but the values in the prediabetics were significantly more variable.

The upper 95% tolerance interval for the normal Indians was calculated. Although no normal Indians exceeded this upper limit by either method, 3% of prediabetics exceeded this limit by the Williamson method compared to 29% by the Siperstein (p=.06). Those prediabetics who exceeded the upper limit of normal had similar glucose tolerance during the OGTT and IVGTT compared to the prediabetics with normal basement membrane thickness.

Long-term follow up of these subjects should answer whether basement membrane thickening precedes glucose intolerance and is, therefore, a predictor of diabetes.

2. Specific Urinary Protein Abnormalities: Preliminary results reveal no differential urinary excretion of proteins by prediabetics compared to normal Indians.

Proposed Course: All results for the basement membrane study will be completed during the next few months. Final analysis will then be undertaken. Glucose tolerance in these subjects is being tested at least every 2 years and plans are presently being made for re-biopsy of these subjects.

Publications:

Aronoff, S.L., Bennett, P.H., Rushforth, N.B., Miller, M., and Under, R.H.: Normal Glucagon Response to Arginine Infusion in Prediabetic Pima Indians. J. Clin. Endo Metab (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 ZM 69,004-03 EFSB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Evaluation of the Prediabetic State in the Pima Indians - Caucasian Controls

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

PI: P.H. Bennett, Chief — EFSB, NIAMDD

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P.J. Savage, Staff Physician — SFSS, NIAMDD

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R.H. Unger, University of Texas Southwestern Medical School at Dallas

M.D. Siperstein, University of California Medical School at San Francisco

J.R. Williamson, Washington University School of Medicine

COOPERATING UNITS (if any)

University of California Medical School at San Francisco
Washington University School of Medicine
Diabetes Branch, NIAMDD

LAB/BRANCH

Epidemiology and Field Studies Branch

SECTION

Southwestern Field Studies Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

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

Nondiabetic Caucasians with a negative family history of diabetes have been examined by means of an oral glucose tolerance test, intravenous glucose tolerance test, and arginine infusion test to measure glucose, insulin and glucagon responses. Muscle capillary basement membrane thickness has also been determined. These data are being compared to those obtained in age-sex-weight matched prediabetic and normal Indian subjects.

PHS-6040 (12-75)

Project Description:

Objectives: Identification of abnormalities in prediabetic individuals has been a major goal of diabetes research in attempting to understand the natural history of the disease.

Tests proclaimed as diagnostic of the prediabetic state include the cortisone glucose tolerance test, time and quantity of insulin release in relation to glucose loads and capillary basement membrane thickness.

These tests were conducted on a group of genetically normal and genetically prediabetic Pima Indians. The very high prevalence of diabetes in the Pima people, while providing a very large group of genetically prediabetic individuals, raised a problem of finding individuals who are truly normal. Genetically normal Pima offspring were selected from genetically normal parents 45 years of age or older. It is conceivably possible, however, that with the high prevalence of diabetes in the tribe, virtually all the Pimas could carry the diabetic genome.

A group of nondiabetic Caucasians with nondiabetic family histories were examined as an additional control group to compare with the "normal" Pimas.

Methods Employed: The study population was selected from nondiabetic Caucasians with nondiabetic family histories who are matched in age, sex and weight with normal Pima volunteers. Each person was admitted to the Clinical Research Center for testing. Tests included an oral glucose tolerance test, arginine infusion, cortisone glucose tolerance test, intravenous glucose tolerance test, a quadriceps needle muscle biopsy and a 24-hour urine collection which is being analyzed for specific urinary proteins by immunodiffusion.

Results:

1. Capillary Basement Membrane Thickness: Results are presently available on 20 of 34 normal Caucasians and 18 of 26 normal Indians. Measurement of MCBMT by the Williamson method revealed a slightly higher mean value in the normal Indians (858±48 Å) compared to Caucasians (788±47 Å). Employing the Siperstein technique, however, the mean MCBMT in the normal Indians (1466±46 Å) was somewhat lower than in the Caucasians (1606±92 Å). Thus there appears to be no consistent racial variation in basement membrane thickness.

2. Specific Urinary Proteins: Preliminary results reveal similar urinary protein excretion in normal Caucasians and normal Indians.

Significance to Biomedical Research and the Program of the Institute: Characterization of the prediabetic state may allow an understanding of the basis of diabetes mellitus. Because of the ability to control many variables it is hoped that this study will allow the demonstration of the earliest lesions of diabetes mellitus.

Proposed Course: Data has been collected on 34 normal Caucasians. Individual's photomicrographs for basement membrane thickness and urine for specific urinary proteins are now being analyzed and prepared for publication. This study has been terminated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69,005-03 EFSB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Evaluation of Basement Membrane Thickness in Diabetic Pima Indians

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

PI: P.H. Bennett, Chief — EFSB, NIAMDD

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P.J. Savage, Staff Physician — SFSS, NIAMDD

R.H. Unger, University of Texas Southwestern Medical School at Dallas

M.D. Siperstein, University of California Medical School at San Francisco

J.R. Williamson, Washington University School of Medicine

COOPERATING UNITS (if any)

Phoenix Indian Medical Center, IHS
Sacaton Indian Health Service Hospital, IHS
University of California Medical School at San Francisco
Washington University School of Medicine

LAB/BRANCH

Epidemiology and Field Studies Branch

SECTION

Southwestern Field Studies Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

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

The relationship of capillary basement membrane thickening to duration of glucose intolerance and the complications of diabetes mellitus are being investigated in the Pima Indians of Arizona. A comparison of the Siperstein and Williamson techniques of analysis is being carried out on these subjects to determine if one method is more sensitive for the detection of diabetes.

PHS-6040
(12-75)

Project Description:

Objectives: Since Siperstein first reported finding thickened muscle capillary basement membranes in diabetics, multiple studies have been performed in an attempt to evaluate his results. Findings have varied remarkably from one investigator to another to the extent that while muscle capillary basement membrane thickening (MCBMT) is generally accepted as characteristic of diabetes, the frequency of its occurrence, its relationship to age, sex, duration of diabetes, and complications of diabetes is now highly controversial.

The contradictions in results appear to be related, at least in part to varying techniques of fixation and measurement employed by different investigators.

A study of MCBMT in normal and prediabetic Pima Indians has been completed. MCBMT in diabetic Pima Indians has not previously been quantitated and it is not known if thickening occurs to the same extent in them as in non-Indians.

The major purposes of the study are:

1. To determine whether Pimas with diabetes develop capillary basement membrane thickening.

2. To determine whether such thickening is related to the duration of carbohydrate intolerance and to the complications of diabetes.

3. To compare capillary basement membranes of diabetic Pimas with the MCBM of normal and prediabetic Pima Indians and normal non-Indians.

Methods Employed: Two groups of diabetics were examined a) long-term diabetics, defined as subjects with fasting plasma glucose $\geq$140 mg/100ml or a two hour post-carbohydrate load glucose $\geq$300 mg/100ml or $\geq$10 years duration with and without retinopathy and b) short-term diabetics, defined as subjects who have had a documented marked deterioration in their oral glucose tolerance over the last 5 years from a two hour post-carbohydrate load plasma glucose $\leq$140 mg/100ml to a fasting plasma glucose $\geq$140 mg/100ml or a two hour post-carbohydrate load glucose $\geq$300 mg/100ml.

Subjects had an oral glucose tolerance test, a quadriceps needle muscle biopsy, a 24-hour urine collection for specific urinary protein determination as well as fundus photographs and direct fundus examination. Those subjects designated as recent-onset diabetics also received an arginine infusion.

Results:

1. Capillary Basement Membrane Thickness: Results are presently available on 25 of the 33 diabetic subjects examined. The mean muscle basement membrane thickness (MCBMT) by the Williamson method in the diabetics of 1832±156 Å (mean ±1 SEM) was significantly greater than that in the normal Indians (852±52 Å). The MCBMT was related to the duration of diabetes ($r=.56$, $p<0.005$) but no relationship to diabetic vascular complications was found.

   The mean MCBMT by the Siperstein method in the diabetics was 3069±191 Å compared to 1466±46 Å in the normal Indians ($p<0.001$). Again, there was a significant positive relationship between MCBMT and duration of diabetes ($r=.53$, $p<0.05$) but not between MCBMT and diabetic vascular complications.

   The 95% tolerance limit for normal Indians was calculated by each method. 68±9% of diabetics exceeded this upper limit for normal by the Williamson method compared to 92±5% by the Siperstein method ($p<0.05$) indicating that in these subjects the Siperstein method was more sensitive in detecting diabetics.

2. Abnormal Urinary Protein Excretion: Results not yet available.

Significance to Biomedical Research and the Program of the Institute: This study is expected to resolve the conflict concerning the optimal method of capillary basement membrane measurement. It will also better define the relationship between the duration of diabetes, diabetic vascular complications and MCBM thickening.

Proposed Course: Basement membrane study will be completed shortly. Analysis of results and preparation of manuscript will then be completed. Analysis of specific urinary protein results is presently underway. This study has been terminated.

Publications:

1. Aronoff, S.L., Bennett, P.H., Rushforth, N.B., Miller, M., and Unger, R.H.: Arginine Stimulated Hyperglucagonemia in Diabetic Pima Indians. Diabetes (in press)

2. Bennett, P.H., Aronoff, S.L., and Unger, R.H.: Evidence for an Insulin-Independent Alpha Cell Abnormality in Human Diabetes. Metabolism (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69,006-06 EFSB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Gila River Indian Community Autopsy Study

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

PI: P.H. Bennett, Chief — EFSB, NIAMDD

OTHER: S.L. Aronoff, Staff Physician — SFSS, NIAMDD
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D. Eatough, Director, Center for Thermo Chemical Studies, Brigham Young University

COOPERATING UNITS (if any)
Indian Health Service
Veterans Administration
Case Western Reserve University

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0.0

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

The postmortem characteristics of Pima Indians of the Gila River Indian Community will be investigated so that findings in subjects with and without diabetes mellitus can be correlated with studies in living subjects.

Postmortem examinations will be performed on members of the Gila River Indian Community whenever possible, regardless of whether the subjects have prior evidence of diabetes. Tissues will be examined by light and electron miscoscopy and the histology related to the clinical findings in the same subjects. Trace metal contents of tissues will be measured to determine relationships of trace metals to diabetes. The data collection will be continued for several years with periodic analyses of the data.

PHS-6040
(12-75)

Project Description:

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.

Research in Progress:

1. Renal Pathology: Diabetic renal glomeruli may be larger than those from nondiabetic kidneys, but little systematic study of glomerular size or variability has occurred. A comparison of renal glomerular size of Pima diabetic and nondiabetic kidneys with those of other groups has been initiated.

	Non Indian Controls n = 18	Pima Nondiabetics n = 11	Non Indian Diabetics n = 20	Pima Diabetics n = 10
Glomerular weight (ng)	224±11.8	397±3.3	453±7.6	738±111.1
Glomerular Diameter (μ)	204± 4.7	236±4.5	249±6.7	267± 12.9
Hydroxyproline (ng/1000 glomeruli)	7.3± 0.4	12.8±1.3	22.3±2.3	36.7± 5.8
Hydroxyproline/ glomerular dry weight (ng/mg)	29.4± 1.1	30.9±2.2	43.4±2.0	46.0± 3.1

Preliminary results from Pima subjects indicate that renal glomeruli were slightly larger than those of white patients, and that diabetic glomeruli were larger than those of nondiabetic Pima controls. An analysis of the hydroxyproline content of these glomeruli and correlation with renal histology is underway.

2. Trace Metals: The high prevalence of diabetes makes the Pima Indians an ideal group for the evaluation of trace metal disease relationships. The analysis of portmortem frozen tissues has been performed. Using atomic absorption and x-ray fluorescent spectroscopy, tissue levels of Cr, Mn, Ca, Mg, Li, Fe, Cu, Zn, and other metals will be described. The liver, pancreas, kidney, fat, hair, aorta and spleen will be analyzed from subsequent autopsies.

The chromium levels in both tissues and hair from diabetic subjects were consistently lower than those from nondiabetics, but the difference was not significant. Additional samples are needed to confirm this analysis.

3. Histopathology of the pancreas: Pancreatic tissue from diabetic and nondiabetic subjects are being examined to determine the number of islets and the relative amounts of α, β and Δ cells of the islets using scanning microscopes and special staining methods.

Significance to Biomedical Research and the Program of the Institute: As more subjects who have been under long term ante-mortem surveillance are autopsied, more precise clinical pathologic correlations will be available for the study of diabetes mellitus than has previously been possible.

Work now in progress to determine renal glomerular tuft size and hydroxyproline content of the glomeruli may help in understanding the pathogenesis of renal disease in diabetics.

Quantification of pancreatic β cells may be used to further define etiologic hypotheses for the role of insulin production and secretion in diabetes mellitus.

The Pima are the first population with diabetes under continued surveillance in which trace metals concentrations have been investigated. Correlation with ante-mortem clinical data and autopsy findings may help elucidate risk factors associated with the development of diabetes, atherosclerosis and other disease entities.

Proposed Course: The systematic collection and cataloging of tissues will continue. As sufficient specimens become available- detailed examinations of various tissues will be made. Further studies of the kidney, including electron microscopy and biochemical quantification of the α and β cells of the islets of Langerhans will be the major activity in the coming year.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69,007-09 EFSB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Prevalence of Diabetes in Indian Populations in the Southwestern United States

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

PI: P.H. Bennett, Chief EFSB, NIAMDD

OTHER: None

COOPERATING UNITS (if any)

None

LAB/BRANCH

Epidemiology and Field Studies Branch

SECTION

Southwestern Field Studies Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

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

The prevalence of diabetes mellitus among various American Indian populations in the Southwestern United States has been determined. Cross-sectional surveys were conducted in collaboration with other health related surveys and in collaboration with other organizations to determine the ranges and variation in prevalence of diabetes mellitus and possible reasons for this variation within the Southwest. The methods used have been similar to techniques used in the detailed studies of diabetes mellitus performed on the Gila River Indian Community.

Data have been obtained from most of the major Indian populations in the Southwest and will be analyzed in conjunction with data from the Gila River Indian Community. The project has been discontinued.

PHS-6040
(12-75)

Project Description:

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 the populations.

Methods Employed: Random samples of total populations have been examined by means of standardized tests of glucose tolerance to enable the prevalence of glucose intolerance to be determined.

Major Findings: Major differences in the prevalence of diabetes among related and unrelated tribal groups have been found.

Significance to Biomedical Research and the Program of the Institute: The findings suggest that the prevalence of diabetes in populations may be environmentally determined as well as being related to the genetic origins of the populations.

Proposed Course: The survey data and genetic markers (blood groups, albumin phenotyping and others) will be analyzed to evaluate genetic and environmental correlation of diabetes among American Indian populations in this region and will be compared and contrasted with those found in similar studies of the Gila River Indian Community. The collection of additional data has been discontinued.

Publications:

Dippe, S.E., Bennett, P.H., Miller, M., Maynard, J.E., and Berquist, K.R.: Coxsackie B4 Virus Infection and Diabetes - Failure to Demonstrate a Causal Association in Man. Lancet i:1314-1317, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69,009-11 EFSB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Natural History of Arthritis and Rheumatism on the Gila River Indian Community

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

PI:	P.H. Bennett, Acting Chief	EFSB, NIAMDD
OTHER:	P.J. Savage, Staff Physician	SFSS, NIAMDD
	S.L. Aronoff, Staff Physician	SFSS, NIAMDD

COOPERATING UNITS (if any)

Stanford University Medical School
University of California, Los Angeles

LAB/BRANCH

Epidemiology and Field Studies Branch

SECTION

Southwestern Field Studies Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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

The development and progression of various forms of arthritis is being investigated in an American Indian population. The patterns of joint disease will be examined to determine if distinctive patterns of degenerative joint disease can be recognized, and if so, their evolution and outcome will be followed. In addition, the prognostic significance of various factors related to rheumatoid arthritis and ankylosing spondylitis will be examined.

The study which includes clinical, radiographic and serologic studies of joint disease is part of a prospective study of the Gila River Indian Community, in which studies of diabetes mellitus and related diseases are being undertaken.

PHS-6040
(12-75)

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, osteoarthritis, 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 Employed:

1. The following tests and examinations will be repeated on all Indians five years of age and 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: The mechanisms of the association between HLA-B27 and ankylosing spondylitis among the Pima Indians has been investigated. Pelvic radiographs of 193 adult Pima Indians (100 males, 93 females), selected at random, but including an excess with diabetes, were read by 2 observers for evidence of sacroiliitis (SI) according to the New York criteria, without knowledge of age, sex, clinical or HLA status.

Thirty-eight subjects (16 males and 22 females; 19.7%) had SI with changes ranging from minimal (Grade II) to total ankylosis (Grade IV). Changes were unilateral in 5 subjects.

HLA-B27 was present in 18% of the Pima population. In those with SI, the antigen occurred in 8 of 16 (50%) males ($p=0.01$) but surprisingly in only 2 of 22 (9%) females. The difference in HLA-B27 frequently between males and females with SI was statistically significant ($p<0.01$). A review of the clinical features of those with SI revealed little difference between the sexes.

Fourteen males know to have ankylosing spondylitis from a previous study (9 or 64%) were B27 positive) were also included for the following analysis. Uveitis was present in 10 males and 5 females. There was a significant

difference in frequency of uveitis between B27 positive (18%) and negative (5%) subjects ($p<0.05$). Uveitis was significantly more frequent in those with SI ($p<0.05$). Again, there was a difference between the sexes; B27 was present in all 4 males with both SI and uveitis but absent in the 2 females with both.

HLA-B27 was approximately 3 times as frequent in Pimas as in Caucasians. The relationship between SI and B27, weaker than in Caucasians, was present in male Pimas only. The higher population frequency of HLA-B27 accounts only in part for the greater frequency of SI in the Pima. Although, like Caucasians, about 20% of Pimas with B27 have SI, the risk of SI in the B27 negative Pima is much greater than in the Caucasian.

Significance to Biomedical Research and the Program of the Institute: The degree of association between HLA-B27 and ankylosing spondylitis in the Pima Indians (and Blacks) compared to Caucasians suggests that there may be other more specific genetic determinants of ankylosing spondylitis which are in linkage disequilibrium with HLA-B27. Well characterized families with B27 negative spondylitis offer the opportunity to search for such determinants utilizing mixed lymphocyte culture techniques.

Purposed Course: The appropriate relatives of families will be investigated by mixed lymphocyte typing techniques in an attempt to identify a spondylitis associated antigen which is more specific for both Indians and Caucasians than the HLA-B27.

Additional studies will also be performed to investigate the purported association between HLA-B27 and hyperostotic spondylitis.

Publications:

Henrard, J-C, Bennett, P.H., and Burch, T.A.: Rheumatoid Arthritis in the Pima Indians of Arizona: An Assessment of the Clinical Components of the New York Criteria. Int J Epidemiol 4,119-126, 1975

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69,012-01 EFSB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cellular Metabolism in Diabetic and Nondiabetic Subjects

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

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P.J. Savage, Staff Physician — SFSS, NIAMDD
W.C. Knowler, Staff Associate — SFSS, NIAMDD
R.H. Williams, Professor of Medicine, University of Washington
J. Goldstein, Professor of Medicine, University of Texas
M. Brown, Assistant Professor of Medicine, University of Texas

COOPERATING UNITS (if any)
University of Washington
University of Texas
Diabetes Branch, NIAMDD

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	0.1

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

Elevated insulin levels in Pima Indian adults compared to weight matched Caucasian controls suggest that cellular changes external to the pancreas may be important in the pathogenesis of diabetes mellitus.

Insulin receptor concentrations will be measured in Indian and non-Indian subjects. Skin fibroblasts have been obtained and measurements of cellular metabolism will be done to search for abnormalities characteristic of diabetes or prediabetes.

PHS-6040
(12-75)

Project Description:

1. To evaluate the number and characteristics of insulin receptors in diabetic and nondiabetic Indians and nondiabetic Caucasians.

2. To evaluate differences in cellular metabolism in diabetic and nondiabetic Indians and nondiabetic Caucasians by the study of fibroblast metabolism.

Objectives:

Insulin receptors: Insulin levels in nondiabetic Pima subjects are elevated when compared to weight matched Caucasian controls. This could be because of a decreased number of cellular insulin receptors, abnormal affinity of Pima cellular receptors for insulin, an abnormality in the insulin secreted by Pima subjects on antibody to the insulin or receptor site or an intracellular abnormality in glucose metabolism. Studies are in progress to measure insulin receptor concentrations and affinity in weight matched genetic normal and genetic prediabetic Indians, diabetic Indians and Caucasians with normal glucose tolerance and no family history of diabetes.

Fibroblast culture and study: Previous reports of decreased cell viability and impaired protein synthesis cultured fibroblasts from diabetics indicate the fibroblast is useful in the study of diabetes. Fibroblast cultures have been established from skin biopsies of genetic normal, genetic prediabetic and diabetic Indians and normal Caucasians. Studies are planned to measure total number of possible cell replications, insulin stimulated glucose incorporation, protein synthesis and lipid metabolism in fibroblasts from these groups.

Significance to Biomedical Research and the Program of the Institute:

Insulin receptors: The measurement of insulin receptors will increase understanding of the hyperinsulinemia described in Pima subjects. Identifing the cause of hyperinsulinemia may be important in understanding the excessive risk of diabetes in Pima subjects.

Fibroblast studies: Detailed studies of fibroblast growth and metabolism should yield important information about possible genetic defects in nonpancreatic cell lines in both the prediabetic and diabetic state.

Proposed Course: Studies of insulin receptors circulating mononuclear blood cells are in progress. Metabolic studies on cultured fibroblasts are about to begin.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69,011-08 EFSB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Experimental and Field Studies of Iodine Metabolism and Endemic Goiter

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

PI:	Robert L. Vought	Chief, Metabolic Diseases Epidemiology Unit	EFSB NIAMDD
OTHER:	Freddie A. Brown	Chemist	EFSB NIAMDD

COOPERATING UNITS (if any)
Laboratory of Nutrition and Endocrinology, Clinical Endocrinology Branch, NIAMDD; Theoretical Statistics and Mathematics Branch, NIMH

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Metabolic Diseases Epidemiology Unit

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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

The search for additional evidence of an association between endemic goiter and the presence of Escherichia coli was continued. This year's findings add additional evidence that goitrous individuals have more antibodies against E. coli than do the nongoitrous. These findings indicate that further research of this relationship can be useful and this course will be continued.

PHS-6040
(12-75)

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. The immediate short-term objectives are to identify and characterize certain microbially-produced goitrogens.

Methods Employed: The current year has been devoted to the development of a quantitative hemagglutination test for the measurement of E. coli antibodies in human serum. Further work on the characterization of the E. coli goitrogen was at a much slower pace because of the lack of personnel.

Major Findings: Previously it was found that the prevalence of endemic goiter is related to water pollution and that E. coli produces a goitrogen. We have now found that goitrous people have more E. coli antibodies than do nongoitrous people living in the same area in Greece.

It is now generally conceded that factors other than iodine deficiency are involved in the etiology of endemic goiter. Our findings indicate that attention to the microbially-produced goitrogens may be a field for fruitful research of thyroid disease.

Work will be continued along current lines with further emphasis on characterization of the E. coli goitrogen.

OFFICE OF SCIENTIFIC AND TECHNICAL REPORTS

Summary

Fiscal Year 1976

The Office of Scientific and Technical Reports (OSTR) prepares the bulk of the regular and special program reports required of the Institute by the Congress, and by the DHEW and the NIH. Staff members also respond to inquiries of all types, including public, press and Congressional.

Generally speaking, this office presents highly technical material in terms meaningful to news media representatives, the Congress, budget officials, the general public, and other interested persons. This is accomplished via press releases, radio and television outlets, audio-visual materials, and related communications apparatus.

Scientific and Technical Reports:

The OSTR staff each year researches and writes a number of special reports and related materials in connection with Congressional appropriation hearings. During FY 1976 special reports were prepared on arthritis, diabetes, digestive diseases, kidney disease, Cooley's anemia, cystic fibrosis and nutrition. The special report on diabetes called for working closely with staff members of several other Institutes, particularly the NHLI and the NEI, which contributed summaries of their respective diabetes-related research activities to the report. Similar efforts were required by the arthritis and kidney disease reports. At the same time, a summary of NIAMDD alcoholism-related research was prepared for inclusion in a special report on alcoholism assembled by the ADAMHA. Related materials prepared by OSTR staff members included the Director's opening statement and the voluminous budget narrative.

In addition, some ten or more NIAMDD intramural and extramural research findings of particular significance were summarized for the publication "Research ADVANCES," which is in its second year and which is offered with the hope that understanding of NIH contributions to biomedical knowledge will be enhanced.

The "weekly report," a brief abstract of a significant intramural or extramural research accomplishment prepared by an OSTR staff member, is used as the basis for many of the regular and special program reports required of the NIAMDD by the Congress, the DHEW and the NIH. These research summaries also are disseminated among science writers, as well as various other publics serviced by the OSTR.

During FY 1975 the weekly reports covered a wide range of disease areas, including (1) the finding that somatostatin has no adverse effect on blood platelet function or the coagulation mechanism in man, although it has been associated with bleeding in baboons; (2) that suppression of glucagon secretion by somatostatin prevents the ketoacidosis associated with insulin deprivation in juvenile diabetes, providing further evidence of a role for glucagon in the development of diabetic ketoacidosis; (3) that low-dose cyclophosphamide plus prednisone is nearly as effective and apparently less toxic than high dose cyclophosphamide in suppressing disease activity in patients with rheumatoid arthritis; (4) that cimetidine, a recently synthesized antihistamine drug, is a potent inhibitor of gastric acid secretion in patients with duodenal ulcer, and is without acute side effects in preliminary clinical trials, and (5) that prolonged treatment of renal osteodystrophy in children via oral administration of a new vitamin D_3 analogue, 1-alpha-OH-D_3, results in both roentgenographic and subjective clinical evidence of improvement.

Freedom of Information Responsibilities

The Freedom of Information (FOI) Act is administered by the Freedom of Information Officer, a staff member of the OSTR. The FOI Officer evaluates all requests by Institute personnel to deny public access to information, advises and counsels Institute staff on all aspects of FOI, and serves as Institute liaison between program officials, general counsel staff and the NIH FOI Officer.

The NIAMDD recommended only one denial during FY 1976, and this request was subsequently denied by the DHEW FOI Officer. The case involved opinions in summary statements named in applications denied in accordance with 5 U.S.C. 552 (b) (5) of the FOI Act and DHEW Public Information Regulations, and salaries of individuals named in applications denied in accordance with 5 U.S.C. 552 (b) (4) of the FOI Act and 45 CFR 5.71 of the DHEW Public Information Regulation (45 CFR Sec. 5.71).

Whereas the OSTR previously maintained an NIAMDD FOI Index, this index has now been subsumed by the NIH FOI Index, which is now maintained by the NIH Office of Communications and covers all BIDs.

Press Activities:

Although the reorganization of the DHEW public information community in FY 1974 resulted in centralizing press and related activities within the NIH Office of Information, the OSTR continues to prepare and issue a representative number of press releases in line with established policy to publicize NIAMDD research activities and programs.

Characteristic press releases during FY 76 included (1) an announcement of the appointment of 11 charter members of the National Commission on Arthritis and Related Musculoskeletal Diseases; (2) a summary of recent findings by NIAMDD and NCI scientists which are contributing to an inter-

national effort that may eventually make possible the manufacture of artificial antibodies, and (3) results of molecular studies which provide concrete evidence that virus genes become incorporated into a normal cell's gene profile and direct abnormal reproduction of the gene material known as DNA (deoxyribonucleic acid). This finding may explain at least one form of cancer.

During FY 1976 OSTR staff members responded to approximately 80 inquiries from representatives of the various news media, and on any number of occasions maintained a "working relationship" with such representatives until culmination of the particular project.

Publications:

As suggested in last year's annual report, the OSTR in FY 76 produced its first publication in a new "Coping With..." series of pamphlets and brochures. The latest addition to the OSTR's list of publications is entitled "How to Cope with Arthritis." Also during FY 76, in a stepped up effort in the publications field, two other new pamphlets - "Facts About Obesity" and "Research Advances in Nutrition" - were produced. Also being distributed is a reprint from "Health" by Benjamin Burton, Ph.D. entitled "Obesity: Our Leading Health Problem." Currently, fact sheets are being prepared on diabetes and hiatal hernia, and several fact sheets on other diseases are being converted into pamphlets.

For the second consecutive Fiscal Year, the OSTR topped the 100,000 mark in the number of publications distributed.

Radio and Television:

Perhaps the most effective means of communicating Institute research activities and programs to the public is through the radio and television media. In recent years, OSTR collaboration with the NIH Office of

Information in this area has resulted in numerous radio and television spot announcements, particularly with regard to the availability of various NIAMDD publications. The public response to this campaign is largely responsible for the OSTR's unprecedented dissemination of printed matter in FY 75 and FY 76. A "Stay-in-School" employee continues to work parttime in the OSTR in order to cope with this continuing public demand for NIAMDD publications.

The OSTR in FY 76 also made arrangements for the appearance of a number of Institute scientists on various radio and television programs, including Drs. Phillip Gorden, Koloman Laki, Jesse Roth, Peter Bennett, and Laurence Miller.

Public Inquiries:

Characteristically, public requests for information are made by either telephone or letter and OSTR staff members respond in kind. Such requests are initiated typically by ill persons and their relatives, students conducting science projects, practicing physicians, and persons who wish to donate money to biomedical research, among others. Quite often these inquiries are rather specific and may require information that is not readily available.

In responding to some 200 or more such inquiries each month, the OSTR staff members often realize that they are doing so at a considerable savings to the time of NIAMDD scientific and administrative personnel.

Congressional Inquiries:

Traditionally, the bulk of the Congressional requests for information directed to the NIAMDD have been handled by staff members of the OSTR. Such requests, initiated either by telephone or letter, may range from a simple request for arthritis literature to a detailed accounting of how much money is spent on diabetes research in a given geographical area, to

a request for a "printout" on all digestive disease-related research conducted and supported by the NIH throughout the world. Invariably, Congressional inquiries are given top priority and are handled with courtesy and dispatch.

During the past few years, staff members of the OSTR have handled approximately 300 Congressional inquiries during each 12-month period of time.

Exhibits:

The OSTR also conducts the NIAMDD program of scientific exhibits used primarily in professional education. Under continued severe travel and exhibit restrictions, the Institute was able to exhibit at only five medical and scientific meetings throughout the country, which were viewed by approximately 35,000 physicians, scientists and paramedical people. Institute exhibits on the artificial kidney, human growth hormone, cystic fibrosis, and general research both intramural and extramural, were shown this year. Various publications and reprints were distributed with these exhibits. Planned for the forthcoming year are a series of exhibits dealing with the Pima Indians of Arizona emphasizing research in diabetes, gallstones, and arthritis.

During the NIH Bicentennial Open House, a special Institute exhibit highlighting half a dozen major research areas went on display, in addition to two other major exhibits. Thousands of visitors thronged NIH during the two day event. Information Office staff members manned the exhibits and distributed many pamphlets and brochures.

OFFICE OF THE DIRECTOR

Summary

Fiscal Year 1976

Dr. G. Donald Whedon, Director
Mr. F. L. Mills, 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 staff 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.

FY-76 saw several important developments affecting the course of the Institute. Perhaps most important were the comprehensive reports issued by the National Commission on Diabetes in December and by the National Commission on Arthritis and Related Musculoskeletal Diseases in April. The Institute plans to implement the recommendations of the Commissions as soon as possible within the limitations of the resources available. The Institute served as the chief administrative support for both Commissions throughout FY-76. Nearly all segments of the Institute were called on during the year to support the Commissions. Institute staff have supplied the commissions with a great deal of research support in addition to the staff support for travel, personnel, comittee management, legislative liaison, etc.

The Contracting Office continued to maintain two major support contracts for the additional administrative requirments which the NIAMDD could not itself supply. Congress passed Public Law 94-278 extending the life of the National Commission on Diabetes through September 1976 and is considering a bill to extend the National Commission on Arthritis and Related Musculoskeletal Diseases until December 31, 1976. These extensions will give the Commissions the opportunity to see that the necessary machinery exists for pursuing the goals of the commissions. The Institute will, of course, continue to support both Commissions to the fullest possible extent.

During the fiscal year the Institute found the highly qualified candidates necessary for the posts of Associate Director for Arthritis, Bone and Skin Diseases and Associate Director for Diabetes, Endocrine and Metabolic Diseases. Dr. Lawrence E. Shulman, Director of the Connective Tissue Division at the John Hopkins Hospital will assume his duties as

Associate Director for Arthritis, Bone and Skin Diseases in July while Dr. Lester B. Salans, Chief of the Endocrine-Metabolism Division in the Department of Medicine at Dartmouth University will come to the Institute in September. These two positions have been vacant since the Institute re-organized under the cluster concept in FY-73. With their occupation the process of re-organization will be culminated.

Institute participation in the US-Japan Cooperative Medical Science Program continues with thirteen ongoing research grants in malnutrition given $856,742 of funding in FY-76. However, the decision of the National Institute of Allergy and Infectious Diseases not to fund any nutrition contracts after FY-76 resulted in contract efforts being reduced to approximately $70,000. As part of the cooperative agreement the Institute co-sponsored the joint meeting and symposium on "Influence of Environmental and Host Factors on Nutritional Factors for Health in Asian Countries" held in Berkeley, Ca., October 28-30, 1975.

The US-USSR Cooperative Program in Arthritis continued under the Institute's coordination in FY-76. In order to implement the US side of the agreement for research in arthritis and related diseases Requests for Proposals were issued in three research areas. As a result two contracts were awarded during the fiscal year. The University of Rochester will conduct studies in Juvenile Rheumatoid Arthritis with Dr. John Baum as Principal Investigator and the New York Medical College will conduct and coordinate a clinical trial of Penicillimine in Rheumatoid Arthritis under Dr. Israeli A. Jaffe. In addition a third contract is being negotiated for immunological studies of Systemic Lupus Erethematosus and it will be awarded during FY-77.

The exchange program between the US and USSR saw several visits of Soviet Scientists during the year. The Fourth Session of the US-USSR Joint Committee for Health Cooperation was held in Betheda October 20-24, 1975 with participants from the Cooperative Program in Arthritis attending. Two Soviet orthopedic surgeons spent several weeks with their US counterparts at the Mayo Clinic and Robert B. Brigham Hosptial during the summer of 1975. Again in March 1976 the Soviets visited American institutions and exchanged information.

Dr. Whedon continued to serve as Chairman of the Life Sciences Committee of the National Aeronautics and Space Administration and also, as a member of the Space Program Advisory Council. In his role as consultant to NASA Dr. Whedon has continued to work closely with PHS/University of California investigators on the NASA bedrest studies for the purpose of developing an effective counter-measure to reduce or prevent the loss of calcium on long space flights. In recognition of his services as principal investigator on the bone-muscle metabolism studies of the Skylab space flights Dr. Whedon was awarded the NASA Medal for Exceptional Scientific Achievement November 18, 1974.

Several important appointments were made during the year. In addition to the Associate Directors previously mentioned, Dr. Martin Rodbell was appointed Chief, Laboratory of Nutrition and Endocrinology and Dr. Terrell L. Hill became Acting Chief, Laboratory of Molecular Biology. The Extramural Program area saw the appointments of Dr. Robert Beall as Metabolism Program Director, Dr. Sarah Kalser as Liver Diseases Program Director and Dr. Gerald Combs as Nutrition Program Director. Miss Merilyn Hiller joined the Office of the Associate Director for Program Analysis and Scientific Communications as a Health Science Administrator serving as the special assistant to the Associate Director.

The volume of contract activity increased significantly with a total obligation budgeted for the year at approximately 9.38 million dollars An important post was vacated during the year, that of contracting officer. Applications were sought for the positions, and it is anticipated that a selection will be made by the close of the fiscal year. Reruitment is also under way for a contract negotiator to assist in handling the growing work load in this area.

Despite the limitations created by these vacancies the contracting office issued RFP's and awarded contracts for cooperative clinical trials of the effectiveness of various drug and other treatments of rheumatic diseases and for a special evaluation study of the effectiveness of hemodialysis in chronic kidney failure. Contract activity in Artificial Kidney, Hormone Distribution, Scientific Communications and augmentation of intramural research remained at basically the same level.

The activities of the Office of Scientific and Technical Reports are included in a separate report which precedes this summary.

In the area of employee training, Basic Ideas in Chemistry continued to be a popular and successful course, with one section each of Parts 1 and V completed in June. The classes stressed a practical understanding of the basic concepts underlying biochemical research. In addition, Mathematics for the Laboratory was offered. Subjects included a review of arithmetic (number systems, basic operations, fractions), charts and graphs, the metric system, exponents, etc. Classes are designed for employees who have little or no formal training in the sciences and empnasize a practical rather than theoretical approach. Upon completion of the training, supervisors are encouraged to utilize their employee's newly acquired skills. The Institute will continue to offer the introductory chemistry and math classes in the fall of 1976 and hopes to offer a continuation of advanced chemistry (VI).

During the year, short-term training opportunities were arranged for 89 secretarial-clerical employees, 38 supervisory managerial personnel and 145 members of the professional staff, as part of this Institute's continuing commitment to the training of it's staff to the fullest possible extent.

Equal Employment Opportunity

J. Harrison Ager continued to serve as the EEO Coordinator and Counselor for this Institute. In that capacity he attended the Annual Career Day at Virginia State College, the Minority Biomedical Science Symposium at Xavier University in New Orleans, and the Minorities in Science Symposium of the AAAS in Boston. Additionally, Mr. Ager participated in local activities directed at equal employment opportunity. These included: speaking before the youth members of the NAACP; attending the Student National Medical Association Conference and participating in the four day workshop held by the Director, DEEO, NIH at the Dulles Marriot.

Mrs. Linnie Sloan began her second year as an EEO Counselor for the Institute. Besides handling ten EEO complaints she participated in formulating recommendations to the Director, NIH for implementing EEO Affirmative Action goals and was chosen as a member of the select committee advising the Director, NIH on current concerns of the EEO community.

The Annual Institute EEO Meeting was held June 1. At that meeting the results of the election for membership to the NIAMDD EEO Adivsory Committee were announced. The laboratories and branches also held their own individual EEO meetings throughout the year. During the period April 18-24 the Institute actively participated in the NIH Secretaries Week. As part of this effort, NIAMDD sponsored a short seminar by David Booker, Booker Associates, on Transactional Analysis.

The Institute published a revised edition of the Affirmative Action Plan which took note of goals accomplished in EEO and addressed current problems within the Institute. The new plan reflects a long period of effort which included the first Institute wide survey on EEO. The Institute also revised the NIAMDD Advisory Committee Working Rules to make that group more responsive to the Institute's EEO needs.