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INTRAMURAL RESEARCH

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Research in the Section on Enzymes is concerned with the following biochemical processes: 1) the cellular regulation of enzyme activities; 2) carrier mediated transport and concentrative uptake of exogeneous compounds by isolated cytoplasmic membrane preparations; 3) the metabolism of amino acids; 4) the mechanism of action of vitamin B_{12} coenzymes; 5) one carbon metabolism; 6) the metabolism of heterocyclic compounds; 7) anaerobic energy metabolism; 8) application of physical methods in the determination of structures of organic compounds; and 9) synthetic studies of organic compounds of biological interest. Results of these investigations are summarized below.

Regulation of Glutamine Synthetase Activity in Escherichia coli.

Previous studies in this laboratory showed that the glutamine synthetase of E. coli and other microorganisms is subject to cumulative feedback inhibition by eight end products of glutamine metabolism.

Results of in vivo studies showed that the feedback inhibitor characteristics of glutamine synthetase is markedly influenced by the state of nitrogen nutrition of the cell. It has now been demonstrated that in vivo modulation of the feedback inhibitor response is achieved by the covalent attachment of adenylyl groups to the enzyme under conditions of nitrogen abundance, and the removal of these groups under conditions of nitrogen starvation. These alterations are mediated by two different enzymatic processes: (1) Adenylylation of the enzyme is effected by a specific glutamine synthetase- ATP adenylyltransferase, which catalyzes transfer of the adenylyl moiety of ATP to the enzyme. The reaction is strongly activated by glutamine and is inhibited by glutamate, and leads to the covalent attachment of up to one equivalent of adenylyl groups per subunit of enzyme; i.e., 12 equivalents per mole. (2) Deadenylylation of the enzyme is catalyzed by a different enzyme that is activated by a-ketoglutaric acid, and by uridine and cytidine di- and triphosphate derivatives, polyuridylic acid, and some ribosomal RNA fractions. The mechanisms of the deadenylylation reaction is still undetermined.

Although adenylylated and unadenylylated forms of the enzyme exhibit marked differences in catalytic behavior, differences in the conformational states that underlie the catalytic characteristics are too subtle to be detectable by several physical measurements. No differences in quaternary or tertiary structures could be detected by electron microscopy nor by measurements of sedimentation velocity, sedimentation equilibrium or viscosity.

Proteolytic digestion of ^{14}C-adenylylated enzyme yielded a decapeptide containing the ^{14}C-labeled adenylyl group. This peptide has the composition: ($asp_2glu_2pro_3gly_1leu_1$ tyr_1). From its stability to hydrolysis by either acid or alkali, and from the characteristic spectral peak at 293 mμ (pH 13) that is elicited by cleavage of the adenylyl ester bond with snake venomen phosphdiesterase, it was established that the adenylyl group is attached through phosphodiester linkage to the tyrosine hydroxyl group. The presence of three proline residues in the decapeptide indicates that this portion of the native enzyme possesses little if any helical configuration and might therefore be in a relatively exposed position in the peptide chain; this may account for its accessibility to the enzymes catalyzing the adenylylation and deadenylylation reactions. The demonstration that tyrosine is the amino acid residue involved in the covalent attachment of the adenylyl group is of special significance since it represents the first instance in which the hydroxyl group of tyrosine has been shown to be functionally involved in esterification of nucleotide derivatives. Whereas it appears likely that similar esterification reactions are involved in the modulation of other regulatory enzyme activities, the generality of the mechanism remains to be determined.

In view of the fact that up to 12 equivalents of adenylyl groups are capable of being attached to each mole of glutamine synthetase, and since the enzyme is composed of 12 apparently identical subunits, it seems likely that each subunit is capable of binding one equivalent of adenylyl groups. It is therefore evident that varying degrees of adenylylation can lead to the formation of molecules having 0 to 12 adenylyl groups per mole. Thus, partial adenylylation can lead to the formation of at least 12 different isoenzymes, differing from each other in the number of adenylyl groups bound per mole; moreover, an even greater number of isomeric forms can exist depending upon the relative orientation of the adenylylated subunits in molecules containing more than one such subunit per mole. In view of these considerations it was of interest to determine if partially adenylylated molecules differ significantly from those predicted on the basis of their average contents of adenylylated and unadenylylated subunits. This knowledge could have a significant bearing on current theories that attempt to explain the regulatory behavior of enzymes on basis of subunit interactions.

To investigate this problem, fully adenylylated and unadenylylated enzyme preparations were mixed together in various proportions to give solutions of enzyme in which the average concentrations of adenylylated subunits varied from 10 to 100%. The catalytic properties of these mixtures were then compared with partially adenylylated enzyme preparations obtained by the random removal of adenylyl groups from a fully adenylylated enzyme by means of controlled hydrolysis with snake venom phosphodiesterase. Thus far, studies of the γ-glutamyltransferase activity indicate that the pH-activity profiles and the extent of activation by Mg^{++} or Mn^{++} for preparations having the same number of adenylyl groups per mole are the same for mixtures of fully adenylylated and unadenylylated enzymes as for the phosphodiesterase treated preparations. These results indicate that, so far as these criteria are concerned, the catalytic activity of each adenylylated and unadenylylated subunit is expressed independently and is not appreciably influenced by the extent of heterologous interaction. This conclusion is valid for the γ-glutamylyltransferase activity with respect

to its divalent ion specificity and its response to pH under the specific assay conditions selected for study. The generality of this conclusion with respect to other parameters is under investigation.

In an effort to understand the cumulative feedback effects of end products, studies on the binding of these compounds and of divalent ions were continued. From equilibrium dialysis studies it is concluded that there are 3 classes of binding sites for Mn^{++}. These include 12 very tight binding sites per mole (one per subunit), 12 slightly less tight sites and up to 48 relatively weak sites. Varying degrees of adenylylation influence the apparent binding constant (k'_A) at the tightest site but have no effect on the other sites. Considered together with various kinetic data and the previously reported relaxation phenomena, the results are consistent with the interpretation that the binding of Mn^{++} at the very tight sites is associated with a conformational change of the protein, and that the ΔF of this change is a function of the state of adenylylation. The 12 sites of intermediate affinity, together with the very tight sites are probably concerned with the catalytic function, whereas some or all of the loose binding sites are involved in gross stabilization of the enzymes.

On the basis of earlier kinetic studies it was concluded that there are probably separate, independent binding sites for each of the 8 different feedback inhibitors. This conclusion is only partially verified by direct equilibrium binding studies, showing that there are 12 equivalent binding sites (one per subunit) for each of the two inhibitors AMP and tryptophan. The binding of these compounds is independent of the presence of Mg^{++} or Mn^{++} and is not influenced by the extent of adenylylation of the enzyme. Although the binding of AMP at subsaturating levels does not appear to be affected by nearly saturating of levels of alanine, serine, histidine or glycine, the binding of AMP is decreased by increasing concentrations of tryptophan; this indicates that some interaction occurs between tryptophan and AMP binding sites. This effect is enhanced by the presence of Mg^{++} but not by Mn^{++}. Interaction between the binding sites for AMP and the substrate sites for glutamate and/or NH_4Cl is also indicated by the fact that high concentrations of these substrates increase the binding of AMP when it is present at subsaturating concentrations. Of special interest was the discovery that no binding of alanine could be detected in the absence of glutamine. This observation is consistant with kinetic studies showing that the inhibition of glutamine synthetase activity by alanine is less at sub-saturation concentrations of glutamate than at saturating levels of this substrate. This observation does not appear to be compatible with the exclusive allosteric binding theory of Monod et al.; it can be reconciled with their non-exclusive binding model if additional assumptions are made. In any case the requirement of substrate for the binding of an inhibitor represents a unique situation and in the present instance makes good sense from the teleological point of view. Under normal conditions the rate of glutamine synthesis may be held in check by moderating the supply of glutamate, whose concentration will be maintained in part through dynamic equilibrium with alanine via transamination. However, under conditions of nitrogen excess high concentrations of both alanine and glutamate will tend to accumulate, but the concerted action of these two amino acids in inhibiting glutamine synthetase activity will guard against an over production of glutamine.

The significance of the adenylylation and deadenylylation reactions in the regulation of glutamine synthetase activity is suggested by the fact that adenylylation of the enzyme is accompanied (a) by its conversion from a form that is intrinsically more catalytically active to a catalytically less active form, (b) by its change from a Mg^{++} dependent to a Mn^{++} dependent form, (c) by an alteration in the pH–activity profile, and (d) by an increase in sensitivity to feedback inhibition by various end products of glutamine metabolism.

The change in divalent ion specificity from a Mg^{++} dependent form to a Mn^{++} dependent form may be particularly significant since activity of the Mn^{++} dependent (adenylylated) enzyme is markedly influenced by the ratio of Mn^{++} to ATP. The activity of the adenylylated enzyme is therefore greatly influenced by the concentration of other nucleoside di- and tri-phosphate derivatives by virtue of their ability to chelate Mn^{++} and thereby influence the ratio of Mn^{++} to ATP.

The ATP: glutamine synthetase adenylyltransferase has been purified 70–100 fold and studies on the mechanism of its action are in progress. Preliminary results indicate that the adenylylation of glutamine synthetase is a two step process in which the adenylyltransferase (AT) acts as an adenylyl group carrier.

1) $ATP + AT \underset{}{\overset{Mg^{++} \text{ or } Mn^{++}}{\rightleftharpoons}} AT\text{-}AMP + PPi$

2) $AT\text{-}AMP + \text{glutamine synthetase} \overset{Mg^{++}}{\rightleftharpoons} AMP\text{-glutamine synthetase} + AT$

The overall adenylylation reaction (reactions 1 and 2) requires the presence of glutamine, is stimulated by sulfhydryl compounds and is inhibited by glutamate, α-ketoglutarate, RNA, tRNA, CTP, UTP, GTP, GDP, UDP, ADP, organic mercurial reagents, inorganic pyrophosphate, orthophosphate and Mn^{++}. The inhibitory effect of Mn^{++} is related to its capacity to bind with glutamine synthetase and thereby render it less susceptible to adenylylation at 37°. The Km for ATP is 1.3 mM; the Mg^{++} form of glutamine synthetase is saturating at 5 mg/ml, and glutamine is saturating at 1 mM.

Reaction 1 is catalyzed in the presence of high concentrations of glutamate or glutamine and either Mg^{++} or Mn^{++}; other nucleoside tri-phosphates will not substitute for ATP. Glutamine synthetase plus glutamine inhibit the reverse of reaction 1, probably by virtue of the capacity of glutamine synthetase to react with AT–AMP in reaction 2. Glutamine is required for reaction 2.

The enzyme system that catalyzes the removal of the adenylyl moiety from adenylylated glutamine synthetase has been purified 4-fold, and has the following characteristics: 1) α-ketoglutarate and a divalent cation (either Mn^{++} or Mg^{++}) are absolutely required for activity; (2) it is inhibited by glutamine; (3) it is markedly stimulated by nucleotides (in order of diminishing activity nucleotides capable of stimulating the deadenylylation are UTP, CTP, ATP, ADP, CDP, poly U, and 5S RNA).

Mononucleotides and other polynucleotides are inactive. (4) Inorganic phosphate increases the stimulation by UTP but has no effect on stimulation by CTP.

It appears significant from the standpoint of cellular regulation that those factors that inhibit the adenylyl transferase are activators of the deadenylylation enzyme system, and vice versa.

These effects and the properties of the adenylylated and unadenylylated forms of glutamine synthetase provide an elegant system control for the regulation of glutamine metabolism. Under conditions of nitrogen excess the reaction sequence α-ketoglutarate→ glutamate → glutamine will be favored and the utilization of glutamine for the biosynthesis of various end products is assured. With excessive accumulation of each end product, feedback control of the first unique step in that pathway will lead to a decrease in glutamine utilization and therefore in an excessive accumulation of glutamine. The high concentration of glutamine will stimulate the adenylyltransferase and inhibit the deadenylylating enzyme system. As a result the catalytically more active unadenylylated glutamine synthetase will be converted to the adenylylated, less active form, which additionally is exquisitely sensitive to cumulative feedback inhibition by multiple products of glutamine metabolism. In this fashion, glutamine directs a decrease in its own biosynthesis. Conversely, with nitrogen starvation, the pool of glutamine would diminish and there would be a relative accumulation of α-ketoglutarate and glutamate. This will lead to inactivation of the adenylyltransferase and an activation of the deadenylylating enzyme. As a consequence, glutamine synthetase is converted to the most active form, which is relatively insensitive to feedback inhibitors. Thus the diminished supply of nitrogen will be more efficiently channeled toward the biosynthesis of glutamine and its metabolites.

Regulation of Glutamine Synthetase Activity in Bacillus subtilis. We previously demonstrated that the glutamine synthetase activities of Bacillus licheniformis and B. subtilis are subject to feedback inhibition by various end products of glutamine metabolism. However, unlike the enzyme from E. coli the inhibition pattern is complex and from kinetic studies it is apparent that there is a high degree of interaction between various inhibitor sites and between inhibitor and substrate sites.

In addition, it was observed that various enzyme preparations of the two Bacillus species showed marked variations in their relative abilities to be activated by Mg^{++} or Mn^{++}. This was assumed to reflect modulation of the glutamine synthetase activity by adenylylation reaction, as was observed with E. coli. In the meantime, results of in vivo studies in Holzer's laboratory indicate that adenylylation of glutamine synthetase does not occur in B. subtilis. It is thus inferred that a different mechanism of control is responsible for the observed variations in divalent ion specificity in this organism. In view of these considerations an effort is being made to purify the glutamine synthetase from B. subtilis in order to investigate the mechanisms of feedback control and to determine the basis of the divalent ion effects. An alanine negative, tryptophan requiring strain of B. subtilis, 10029, was selected for these studies since the concentration of glutamine synthetase in this strain is abnormally high. A further 3-5 fold

increase in the concentration of this enzyme was obtained by utilizing glutamate rather than ammonia as a source of nitrogen for growth.

Using conventional isolation techniques a 25-30 fold purification of the enzyme has been obtained, yielding a preparation that is relatively homogeneous as judged by polyacrylamide gel electrophoresis and sedimentation in the ultracentrifuge in the presence of dithiothreitol, EDTA and 7 M urea. In the absence of these reagents the enzyme exists in various states of aggregation as disclosed by disc gel electrophoresis and sedimentation analysis. The isolated enzyme is most active as measured in the standard biosynthetic assay in the presence of Mn^{++}; it is considerably less active with Mg^{++}. Preliminary evidence suggests that Mg ATP or Mn ATP may serve as substrates, but a separate Mn^{++} specific site is required for maximal activity. The activity is stimulated 250% by sulfhydryl compounds and is enhanced by high ionic strength. The latter effect is probably associated with increased stability of the enzyme under the assay conditions, and is most marked when Mg^{++} is the divalent cation. Further experiments will be directed toward a detailed analysis of the feedback inhibitor characteristics and the nature of the divalent ion effects.

Metabolic Regulation and Mechanism of Action of Phosphoribosylpyrophosphate Synthetase. The biosynthesis of 5-phosphoribosyl-1-pyrophosphate (PRPP) is the point at which ribose-5-phosphate is removed from the oxidative pentose cycle and utilized for the biosynthesis of purine and pyrmidine nucleotides, tryptophan, histidine, and pyridine nucleotides. Previous investigations in this laboratory have shown that the PRPP synthetase in Salmonella typhimurium is subject to regulation by an additive type of feedback inhibition by numerous end products of PRPP metabolism. In continuing studies, the possibility that the concentration of PRPP synthetase is regulated by repression has been investigated. Growth of S. typhimurium in the presence of high levels of end products derive from PRPP or starvation for carbon or phosphate source had only slight effect on the level of PRPP synthetase as compared to the activity of cells grown on a glucose-salts medium. De-repression of the histidine operon with concomitant over production of histidine, and hence also of PRPP, had no effect on the specific activity of PRPP synthetase. Thus, either PRPP synthetase is a constitutive enzyme, or the nutritional state of the organism has little effect on the level of the co-repressor.

In order to investigate the molecular basis of additive feedback control and to determine the biochemical mechanism of PRPP synthesis, the PRPP synthetase has been isolated from extracts of S. typhimurium. The purified synthetase requires Mg^{++} or Mn^{++} for activity. The substrate saturation curve for Mg-ATP is sigmoid, but is converted to a hyperbolic curve by excess Mg^{++} or Mn^{++}. Kinetic studies show that both metal ions cause an increase in the affinity of enzyme for the Mg-ATP complex and that there are at least two sites with different affinities for Mg-ATP. The activation by Mg^{++} at low levels of Mg-ATP is potentially several hundred fold. This could provide the basis of an important regulatory mechanism, but its significance has not been determined.

PRPP synthetase has been found to catalyze two exchange reactions: (1) AMP:ATP exchange in the absence of ribose derivatives, and (2) ribose-5-phosphate:PRPP exchange in the absence of adenine nucleotides. The AMP:ATP exchange reaction requires enzyme, Mg^{++}, and PRPP as well as ribose-5-phosphate. AMP at 10^{-4} M and below markedly stimulates the ribose phosphate: PRPP exchange; at higher levels of AMP the reaction is very nearly completely inhibited. These exchange reactions suggest that the PRPP synthetase reaction proceeds via an enzyme-pyrophosphate intermediate:

$$\text{Enzyme} + \text{ATP} \rightleftharpoons \text{Enzyme PP}_i + \text{AMP}$$

$$\text{Enzyme PP}_i + \text{R-5-P} \rightleftharpoons \text{Enzyme} + \text{PRPP}$$

The formation of an enzyme-pyrophosphate has been demonstrated directly by incubating the enzyme with ATP-γ-^{32}P and separating the products on Sephadex G-25. Label from ATP-γ-^{32}P was incorporated into the enzyme; control experiments with ATPUl-^{14}C showed that no more than 2-3% of this labeling could have resulted from binding of intact ATP. When ribose-5-phosphate was included in the incubation mixture, virtually no ^{32}P labeled enzyme was obtained, which suggests that the enzyme-pyrophosphate transfers the pyrophosphate to the acceptor. These studies indicate that it should be possible to isolate and chemically characterize the enzyme-pyrophosphate intermediate.

The effects of inorganic phosphate on PRPP synthetase are complex. Removal of phosphate by dialysis brings about a total, irreversible inactivation of the enzyme, both in the overall reaction and in the exchange reactions. Simply lowering the phosphate concentration by dilution partially inactivates the enzyme. Phosphate is required for the synthesis of PRPP, yet the exchange reactions are inhibited strongly by phosphate with a concentration dependence which is very similar to the dependence of phosphate stimulation on concentration. Thus, phosphate seems to have a role in maintaining the structural integrity of the protein, as well as a role in the enzyme catalysis.

Carrier Mediated Transport and Concentrative Uptake of Exogeneous Compounds by Isolated Cytoplasmic Membrane Preparations

Concentrative Uptake of Sugars. Previous work from this laboratory reported the isolation of a membrane preparation from E. coli which, in the absence of soluble proteins catalyzes the concentrative uptake of proline as well as facilitated diffusion of glycine and its ultimate conversion to phosphatidylethanolamine.

It has now been established that these isolated membrane preparations are able to catalyze the uptake of various sugars. This uptake involves phosphorylation of the sugar, via the phosphotransferase system which was shown by Roseman et al. to catalyze the transfer of phosphate from P-enolpyruvate to various carbohydrates according to the following reactions:

I. P-enolpyruvate + HPr $\xrightleftharpoons{\text{Enz. I, } Mg^{++}}$ Pyruvate + HPr-P

II. P-HPr + Sugar $\xrightarrow{\text{Enzyme II. } Mg^{++}}$ Sugar-P + HPr

HPr, a heat stable, low molecular weight protein, and Enzyme I are predominantly soluble proteins, whereas Enzyme II is membrane bound.

Evidence for the role of this system in the uptake of a-methylglucoside by isolated membrane preparations of E. coli is as follows: 1) P-enolpyruvate is specifically required for the uptake of the glucoside. (2) During the uptake of a-methylglucoside there is a stoichiometric relationship between the disappearance of ^{32}P-enolpyruvate from the medium and the formation of a-methylglucoside-^{32}P: (3) Membranes prepared from E. coli GN-2, a mutant lacking Enzyme I of the P-transferase system, are unable to take up significant quantities of a-methylglucoside. (4) The rate of uptake of a-methylglucoside by membranes is markedly stimulated by the addition of purified preparations of HP and Enzyme I.

Results from dual labeling experiments indicate that the phosphorylation of glucose by the P-transferase system is an integral part of the permeation process itself and does not take place after the glucose has already penetrated the membrane. This is indicated by the fact that when ^{3}H-glucose and p-enolpyruvate are added externally to suspension of membranes that were pre-loaded with ^{14}C-glucose (in the absence of P-enolpyruvate), intramembranal accumulation of ^{3}H-glucose-P occurred more rapidly than the accumulation of ^{14}C-glucose-P. Thus the externally added glucose was phosphorylated more rapidly than free glucose in the intramembranal pool. Participation of the phosphorylation reaction in the permeation process is further indicated by the fact that the uptake and phosphorylation of a-methylglucoside exhibit saturation kinetics with an apparent Km of about 4×10^{-6} M, whereas the rate of appearance of free a-methylglucoside in the intramembranal pool is independent of the presence of P-enolpyruvate: the rate is proportional to the external concentration of a-methylglucoside over a tremendous range, and even at relatively high external concentrations (10^{-4} M) is much slower than the rate of accumulation of the phosphorylated derivative in the presence of P-enolpyruvate.

In collaboration with Dr. Roseman's group at Johns Hopkins University, the effects of HPR and Enzyme I on the uptake of a-methylglucoside by isolated membranes was studied in greater detail. In the absence of added HPr and Enzyme I the initial rate of uptake is proportional to the P-enolpyruvate concentration of the range of 0 to 0.1 M However, in the presence of added HPr and enzyme I the rate of uptake is a sigmoidal function of the P-enolpyruvate concentration and is maximal at 10^{-3} M. In either case, the a-methylglucoside taken up is nearly all present as the phosphorylated derivative.

Of special interest was the finding that sodium fluoride inhibits the stimulation of α-methylglucoside uptake by HPr and Enzyme I at low concentrations of P-enolpyruvate; nevertheless, under these conditions phosphorylation of the glucoside continues, but the sugar phosphate appears in the external medium rather than within the membrane vesicles. Whereas these results indicate that the membrane-bound Enzyme II is capable of releasing sugar-P either into the membrane vesicles or into the external medium, the capacity of sodium fluoride to influence the polarity of the process is still obscure.

Studies of the effect of temperature on α-methylglucoside uptake and phosphorylation by membranes prepared from a variety of strains of E. coli, Salmonella typhimurium, B. subitilis have demonstrated that the membranes begin to leak sugar phosphates when the temperature is raised above a certain point. Furthermore the point at which they begin to leak differs with membranes prepared from different strains of E. coli and in membranes derived from cultures of B. subtilis grown on either minimal or enriched media. The temperature optimum for phosphorylation (regardless of transport) is at about 46° and is about the same for each of these membrane preparations. Finally, the leakage of sugar-P is due to a rapidly reversible increase in membrane permeability at the higher temperatures. Experiments recently carried out with Drs. Roy Vagelos, David Silbert, and Mr. Frank Ruch at Washington University, St. Louis indicate that the unsaturated fatty acids present in the phosphatides of the membranes may play an important role in these leakage phenomena. Using membranes prepared from their mutant which requires an unsaturated fatty acid for growth, membranes were prepared from cells grown on oleate (vaccenate is the normal predominant unsaturated fatty acid in E. coli). These membranes were demonstrated to leak at 2-3 times the rate of wild type membranes and furthermore, when examined under the electron microscope, were found to contain a large number of open vesicles.

Metabolism of Amino Acids

Reactions and Enzymes Mediating the Biological Transfer of Sulfur Between Cysteine and Homocysteine. Genetic evidence in Neurospora as well as analogy to bacteria suggested the sequence: sulfide → cysteine → cystathionine → homocysteine → methionine in which the key process is trans-sulfuration from cysteine to a four carbon amino acid. Previous work in this laboratory established that: 1) O-acetylhomoserine is the four carbon precursor, and 2) there is an enzyme in Neurospora (missing in a methionine auxotroph) that cleaves cystathionine to homocysteine. Initial attempts to show the missing step, enzymatic synthesis of cystathionine from cysteine and O-acetylhomoserine, were not successful. In fact, the demonstration that homocysteine could be synthesized in vitro by the reaction, sulfide + O-acetylhomoserine → homocysteine, prompted speculation that cystathionine is not a necessary intermediate in methionine biosynthesis. However since this would not explain the in vivo requirement for the cystathionine cleavage enzyme, the search for an enzyme in Neurospora that catalyzes the synthesis of cystathionine from cysteine and O-acetylhomoserine, was continued. The activity of this enzyme is sufficient to account for methionine biosynthesis in vivo. Failure to detect this enzyme previously was due to the fact that it is extremely labile and did not survive the procedure used in prepairing cell free extracts. The enzyme is quite

9

specific for O-acetylhomoserine, showing little or no activity with homoserine or O-succinylhomoserine (the corresponding intermediate in bacteria).

Mutants of two unlinked genes were found to lack cystathionine synthase. These mutants, me-3 and me-7, were previously shown to be able to grow on cystathionine but not on cysteine plus O-acetylhomoserine. This is particularly interesting since the corresponding enzyme in Salmonella is controlled by one gene and appears to be composed of identical subunits. When extracts from the two types of Neurospora mutants were mixed activity was restored. This indicates that there are two gene products which can be recombined in vitro. The nature of these two components is not known except that they are both macro-molecules. Enzyme activity is lost upon dilution suggesting that the two components may function as a complex.

A pyridoxal phosphate enzyme, O-acetylhomoserine sulfhydrylase, which forms homocysteine from O-acetylhomoserine and sulfide has been purified 500 fold from Neurospora. It is very specific for O-acetylhomoserine, but differs from cystathionine synthetase in that it reacts with sulfide and not cysteine. Cystathionine synthase from Salmonella reacts with either cysteine or sulfide. The sulfhydrylase is present in both me-3 and me-7 mutants and addition of purified wild type sulfhydrylase does not restore cystathionine synthase activity to either type of extract. Therefore, the function of the sulfhydrylase remains unknown, although the present evidence does not completely rule out a relationship to cystathionine synthase.

When Neurospora is grown in a media containing excess methionine an inhibitor of cystathionine synthase accumulates. The inhibitor has not been identified but is not methionine itself and might be S-adenylhomocysteine.

Purification and Properties of Salmonella β-Cystathionase. β-Cystathionase, catalyzes the reaction: Cystathionine + H_2O → Pyruvate + Homocysteine + NH_3. Purification of this enzyme from Salmonella extracts has been undertaken because of its possible usefulness in assaying for cystathionine, and also to investigate the possibility that it is inhibited by rhizobitoxine, a toxin of bacterial origin that causes chlorosis in plants. The enzyme was purified 300 fold from derepressed Salmonella Me A 15. The apparent Km for cystathionine is 3×10^{-4} M. Cystine is also a good substrate but cysteine is a poor one. The enzyme has a sharp pH optimum at pH 8.5.

Although the structure of rhizobitoxine is not known, it resembles cystathionine in several respects and prevents the growth of Salmonella. In collaboration with Lowell Owens from the U.S. Soils Laboratory, U.S.D.A. at Beltsville, Md., it was shown that rhizobitoxine is a potent inhibitor of the purified β-cystathionase. Kinetic studies show that the inhibition is a mixed noncompetitive type and that the apparent K_i for the toxin is 2×10^{-8} M.

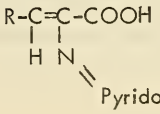
The mechanisms of γ-elimination and γ-replacement reactions catalyzed by cystathionine γ-synthetase from Salmonella typhimurium. The ability of cystathionine γ-synthetase to catalyze reactions in which covalent bonds to the γ-carbon atom of certain amino acids are formed and broken makes it unique among pyridoxal phosphate enzymes. This enzyme therefore provides an interesting model system for studies on the mechanism of pyrodoxyl phosphate potentiated γ-elimination and γ-replacement reactions.

The reaction, O-succinylhomoserine + H_2O → succinate + α-ketobutyrate + NH_3 is an example of a γ-elimination reaction catalyzed by cystathionine-γ-synthetase. To determine whether hydride transfer or proton labilization generates the unsaturated intermediate which must be formed as an intermediate, this reaction was carried out in deuterium water. The resulting α-ketobutyrate, isolated as a dinitrophenylhydrazone and assayed for deuterium by nuclear magnetic resonance spectroscopy, contained 1.0 atoms per mole excess deuterium in the β position and 0.2 in the γ position. Although this result does not rule out hydride transfer completely, it more likely indicates a protolytic mechanism in which the enzyme shields a labilized proton from exchange with solvent water. The deuterated α-ketobutyrate also was degraded to optically active sodium propionate, indicating that addition of a proton to the β-position of an unsaturated intermediate, a relatively late step, is enzymatic. In addition to catalyzing the overall reaction:

O-succinylhomoserine + L-cysteine → L-cystathionine + succinate (2)

the synthetase also catalyzes the γ-replacement reaction with cysteine (i.e., ^{35}S-Cysteine + cystathionine $\rightleftharpoons$ ^{35}S-cystathionine + cysteine). Finally experiments carried out in tritiated water show that the enzyme catalyzes exchange of the α-hydrogen and one stereospecific β-hydrogen in each half of the cystathionine molecule with solvent hydrogens. The finding that the exchange of tritium into the 4 carbon chain of cystathionine is 500 times faster than the cysteine-cystathionine exchange reaction indicates that γ-elimination occurs by removal of cysteine, the γ-substituent. This interpretation is confirmed by the finding that the enzyme catalyzes hydrogen exchange between water and α-aminobutyrate, an amino acid with no γ-substituent.

The enzyme also catalyzes tritium exchange between water and α- and β- positions of three carbon amino acids such as serine, cysteine and alanine. Whereas the enzyme catalyzes β-elimination from succinyl serine and cystathionine, the β-hydrogen exchange must be a side reaction rather than part of the β-elimination mechanism. The resulting dead end intermediate, might account for a transient 465 mμ absorption band when the enzyme is saturated with succinylserine and also for the slowing of β-elimination from this substrate which occurs during the first 3 or 4 minutes of the reaction.

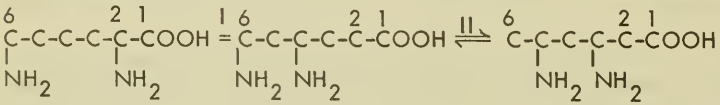


Further studies on the changes in the enzymes absorption spectrum have been undertaken. Both succinylhomoserine and succinylserine cause transient quenching of the enzyme's 420 mμ absorption band and the appearance of a new band at 480 mμ, but only succinylserine gives the new band at 465 mμ. Alanine and α-amino-butyrate also quench the 420 mμ band but give rise to no new bands. Of special interest was the finding that there are no spectral changes when both cysteine and succinylhomoserine are present. After all the cysteine is consumed in the synthesis of cystathionine (reaction 2), the changes characteristic of γ-elimination occur if succinylhomoserine is present in excess. Similar studies of changes in the enzymes excitation and emission spectra have been initiated.

Lysine Fermentation. Earlier studies in this laboratory showed that the fermentation of lysine by Clostridium sticklandii and related organisms is described by the overall equation:

$$\text{lysine} \xrightarrow{+2H_2O} \text{butyrate} + \text{acetate} + 2\,NH_3$$

From isotope studies it was established that the butyrate and acetate were produced by two alternate pathways of metabolism: in one pathway (A), acetate and butyrate are derived by a mechanism involving cleavage of lysine between the 2 and 3 carbon atoms; in another pathway (B) they could be formed by cleavage between carbon atoms 4 and 5. In recent years the first pathway has been studied in soluble extracts and has been shown to involve the intermediary formation of two different diamino acid derivatives produced in two consecutive amino group migrations illustrated by reactions I and II in Scheme A.



α-L-lysine β-L-lysine 3,5-diaminohexanoate

SCHEME A

$$2NH_3 + \overset{6}{C}H_3CH_2CH_2C00H + \overset{2}{C}H_3\overset{1}{C}00H$$

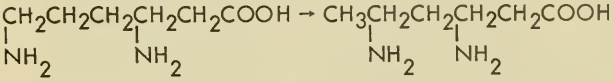
The overall conversion of lysine to acetate and butyrate by pathway B has not been demonstrated in cell-free extracts, presumably due to destruction of one or more labile catalyst. However, a new B_{12}-coenzyme dependent reaction has been discovered, which is thought to be the first step in pathway B. α-D-Lysine is the substrate for this new reaction, which involves migration of the amino group on the number six carbon 5, producing 2,5-diaminohexanoate. Properties of the enzyme system catalyzing this reaction are summarized in the section on "The Mechanism of Action of Vitamin B_{12}-Coenzymes".

Proline reduction. Studies on the nature of the electron transfer processes involved in the reduction of proline to Δ-aminovaleric acid by enzymes from Clostridium sticklandii and Clostridium lentoputrescens have been continued. The ability to utilize DPNH, TPNH or dithiothreitol as electron donors for the reduction of proline was previously reported. Partial purification of the reductase activity by chromatography on DEAE cellulose and fractional precipitation with ammonium sulfate, yielded preparations in which the ability to use all three electron donors was maintained. Such preparations possess diaphorase activity catalyzing the reduction of triphenyltetrazoline chloride by DPNH or TPNH, and existence of a common electron carrier in dye reduction and proline reductase is indicated by the capacity of tetrazolium dye to inhibit proline reduction. By means of filtration through a column of agarose A, the ratio of DPNH-linked and dithiothreitol-linked proline reductase activities was changed from 0.66 to 0.033. This was accompanied considerable purification of the proline reductase activity, but also polymerization of the enzyme.

The possibility that ATP synthesis is coupled with the reductase of proline was investigated with crude dialyzed extracts of C. sticklandii and C. lentoputrescens using a DPNH generating system consisting of either ethanol or isoproponal together with alcohol dehydrogenase. ^{32}Phosphate esterification (0.4 moles per mole of proline reduced) was observed with extracts of C. lentoputrescens when ethanol was used as the electron donor, but no esterification was detected when isopropanol was the electron donor. When ethanol was the electron donor, acetate was produced in an amount equal to the ATP formed; this suggests that the observed phosphorylation may be due to a side reaction yielding acetylphosphate.

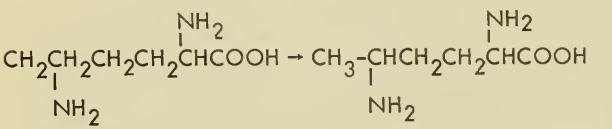
Studies on the mechanism of amino acid degradation might be greatly facilitated by the use of biochemical mutants that have metabolic defects with respect to amino acid fermentation. However, until now, the isolation of mutant strains of obligately anaerobic bacteria has been impracticable because of the technical difficulties involved in the use of classical plating techniques for the selection of the desired mutants. With availability of the anaerobic laboratory, these technical problems are circumvented. Therefore, studies have been initiated to obtain mutants of C. sticklandii whose energy metabolism is restricted to the fermentation of various pairs of amino acids. Following treatment with the powerful mutagen, nitrosoguanine, the cells were plated on a complex medium and some of the small colonial forms that developed were isolated; these were assumed to be mutants having defects in metabolism and therefore are probably defective in their ability to ferment one or more amino acid in the growth medium. Of the survivors from nitrosoguanine treated suspensions, 40-50% were small colonial forms. Several of the isolated strains showed marked deficiencies in their capacity to ferment lysine or proline, or various combinations of these. These preliminary results are very encouraging and demonstrate the feasability of doing mutant research on obligately anaerobic bacteria in the anaerobic laboratory. This appears to open up new approaches to the solution of many interesting problems of intermediary metabolism that can best be studied in obligately anaerobic microorganisms.

Isomerization of α-D-Lysine and β-lysine. It was previously reported that migration of the amino group from carbon 6 to carbon 5 of β-L-lysine to produce 3,5-diaminohexanoate (reaction 1) is a B_{12}-coenzyme dependent step in the fermentation of lysine by Clostridium sticklandii.



(1)

A second B_{12}-coenzyme dependent reaction in the overall lysine fermentation has now been discovered. In this reaction, α-D-lysine is converted to the 2,5-diaminohexanoate derivative (reaction 2)



(2)

The α-D-lysine mutase system that catalyzes this reaction has been partially purified and, like the β-L-lysine mutase system it is inhibited by intrinsic factor, activated by B_{12}-coenzyme, and requires ATP, a sulfhydryl reducing agent, Mg^{++} and is stimulated by FAD. Unlike the β-lysine mutase system, pyruvate does not activate the α-D-lysine mutase system. Further similarity between the two systems is evident from the fact that they both consist of two separable proteins. One of these, a sulfhydryl protein, seems to be interchangeable with the sulfhydryl protein component of the β-lysine mutase system. The other, an acidic red cobamide protein fraction, is very similar in properties to the β-lysine mutase cobamide protein. Whereas preparations of the β-lysine mutase cobamide protein plus the sulfhydryl protein component catalyze only the β-lysine mutase reaction, all of the cobamide protein fractions active on α-D-lysine are also active on β-L-lysine. It remains to be determined if there are two essentially different cobamide proteins or whether there is only a single cobamide protein whose specificity for the different amino acid substrates is determined by other factors (viz. by its interaction with low molecular weight compounds). Experiments to determine if the two cobamide protein fractions are identical are in progress.

Studies on mechanism of action of B_{12}-coenzyme in the β-lysine mutase reaction, continued in cooperation with J. Retey in Zurich, have clearly established that the coenzyme serves a hydrogen carrier for the hydrogen that is removed from carbon 5 and replaced by the amino group. This hydrogen then is transferred back to carbon 6 and appears in the methyl group of the 3,5-diaminohexanoate product. This role as a hydrogen carrier is common to that established for B_{12}-coenzyme in 4 other known reactions.

The possible significance of the α-D-lysine mutase and β-L-mutase reaction in the overall lysine fermentation by C. sticklandi is discussed in the section on amino acid metabolism.

Ethanolamine Deaminase. Studies on the mechanism of action of the B_{12}-coenzyme dependent deamination of ethanolamine as catalyzed by Clostridial ethanolamine deaminase were continued. It was previously established that the deamination reaction involves substitution of the amino group on carbon 2 of ethanolamine with a hydrogen atom derived from the carbinol carbon of the substrate. Experiments with tritiated substrate have shown that the B_{12}-coenzyme mediated transfer of hydrogen from the carbinol carbon to the 2-carbon atom is stereo-specific with respect to which of the two hydrogen atoms on the carbinol carbon are transferred.

The previous report that certain B_{12} analogs could be substituted for B_{12}-coenzyme as co-catalyst in the deaminase reaction could not be confirmed. The slight activity of the anlogs was shown to be due to the presence of trace quantities of B_{12}-coenzyme in the crystalline vitamin B_{12} preparation used in the preparation of the analogs. The catalytic activity attributable to contamination of various B_{12}-derivatives with coenzyme would ordinarily go undetected but it became manifest when studies were made with substrate levels of enzyme and B_{12} compounds. When the contaminating coenzyme was rigorously removed, it could be shown that all analogs previously tested, as well as several others, are inhibitors of the deaminase reaction.

The capacity of B_{12}-analogs to induce tight binding of ethanolamine to the enzyme was confirmed. Results of kinetic studies on the inhibition by B_{12}-analogs suggests that the enzyme exists in two forms, which differ in their maximum velocity and in their affinity for various inhibitors. In the presence of an inhibitor the enzyme is converted from one form to another.

Binding of coenzyme and of hydroxy B_{12} to the enzyme leads to a change in the circular dichroism of the cobalamines. Titrations by this technique showed that the enzyme possessed 2 binding sites for these compounds. Kinetic experiments confirmed this result and in addition showed that each binding site represented a separate and independent active site.

Aerobic photolysis of alkyl cobamides leads to the production of hydroxy B_{12} via the homolytic cleavage of the carbon-cobalt bond followed by oxidation of the resulting B_{12r}. Preliminary studies with a number of enzyme-cobamide complexes have shown that aerobic photolysis leads to products other than hydroxy B_{12}. In particular, ESR studies indicate that the enzyme is capable of stabilizing a paramagnetic species produced by the photolysis of both methyl B_{12} and coenzyme. There is preliminary evidence that in the presence of acetaldehyde, the enzyme is capable of splitting the coenzyme at the carbon-cobalt bond.

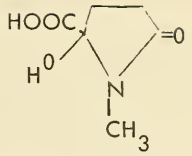
Role of B_{12}-Coenzyme in the Fermentation of Nicotinic Acid. A role of B_{12}-coenzyme in the fermentation of nicotinate by a certain strain of Clostridia was suggested from the demonstration that this organism contained exceptionally high concentrations of a benzimidazole cobamide type coenzyme and also by the fact that intrinsic factor partially inhibited the conversion of nicotinic acid to its ultimate fermentation products by cell free extracts. Evidence is now available showing that the coenzyme dependent step is concerned with the overall conversion of an intermediate a-methyleneglutarate to a carbonyl containing derivative, which has not been identified. Conversion of a-methyl-eneglutarate to the carbonyl derivative is inhibited by intrinsic factor; this inhibition can be overcome by addition of demethylbenzimidazole cobamide coenzyme. The coenzyme also activates the uninhibited reaction.

One Carbon Metabolism

Synthesis of acetate from CO_2. Previous studies have shown that Comethyl cobalamin is an intermediate in the conversion of CO_2 to acetate by cell free extracts of Clostridium thermoaceticum. Moreover, two separate proteins, A and B fractions, in addition to ferredoxin and pyruvate plus CoA were shown to be involved in the conversion of methyl cobalamine to acetate. The discovery of a strong pyruvate dehydrogenase activity in protein fraction A prompted further experiments to determine if this activity was involved in the methylcobalamin to acetate conversion. Purification of the pyruvate dehydrogenase activity was facilitated by the use of Evan's blue as an electron acceptor. This electron acceptor has advantages over other electron acceptor dyes since the reduced form is not autooxidizable. Using partially purified preparations of the pyruvate dehydrogenase it was shown that a mixture of TPNH and DPN could replace pyruvate and CoA as electron donors for dye reductase. The curious requirement for both TPNH and DPN was not observed when methylviologen replaced Evan's blue as the electron acceptor. Following dialysis against KBr this diaphorase activity was lost and could be restored by Mn^{++} and FAD and that then the requirement of DPN for Evan's blue reduction was lost.

The partially purified Evan's blue reductase system does not convert Co-methyl-cobalamin to acetate nor can it replace completely either fraction A or fraction B. However, when systems capable of carrying out the conversion at low rates are supplemented with the Evan's blue reductase fraction, considerable stimulation is observed. This suggests that the reductase activity may be one of several components in the overall conversion of methylcobalamin to acetate.

Metabolism of Methylamine. As part of a general program on the metabolism of one carbon compounds, studies on the metabolism of methylamine by soluble extracts of Pseudomonas MA have been continued. Last year it was reported that the product of an enzyme catalyzed reaction between methylamine and a-ketohlutarate was tenatively identified as the N-methylamide of 2-hydroxyglutarate. This product has now been shown definitely to be N-methyl-5-hydroxypyroglutamine acid:



Its structure was deduced from its elemental composition, and from mass spectroscopic measurements, and was confirmed through its synthesis by the oxidation of N-methyl-glutamine with L-amino acid oxidase. Various lines of evidence indicate that the formation of the pyroglutamic derivative does not involve the enzyme system reported previously that catalyzes the synthesis of N-methylglutamate from methylamine and glutamate.

Other studies have shown that the methyl group of methylamine is incorporated into the α or β carbon of alanine and to a smaller extent into other amino acids and apparently neutral substances. Work is in progress to identify the unknown compounds and to establish the mechanism by which alanine is formed.

Methane Fermentation. The reduction of CO_2 to methane is a problem of considerable biochemical interest since it offers a unique opportunity to investigate an aspect of one carbon metabolism that may provide information of more general significance. Previous studies showed that a methyl-B_{12} derivative is an intermediate in the reduction of CO_2 to methane by enzyme preparations of Methanosarcina barkeri, and that the overall reduction requires ferredoxin, ATP, CoA, an unidentified thermostable co-factor. Work on this project has been limited due to the departure of the principle investigator.

In general progress on this problem has been hampered by the exterme lability of some enzyme components to autoaxidation and to technical difficulties encountered in the design of experiments and in the assay of catalytic activities. These difficulties are now partly resolved with the availability of the anaerobic laboratory. Using this facility, an assay method for methane biosynthesis by cells and extracts of Methanosarcina barkeri has been devised that can be carried out as simple test tube experiments instead of using a more cumbersome manometric apparatus. The oxygen-sensitive enzymes and other reaction mixture components are added, in the anaerobic laboratory, to test tubes which are then closed with serum parts. During incubation of these samples in the ordinary laboratory, aliquots of the gas phase are withdrawn with syringes and assayed for methane. In this system, preliminary results indicate that, in the absence of H_2, extracts as well as frozen cells can ferment acetate to $CH_4 + CO_2$. A combination of extract plus a small aliquot of cells gave much more than additive activity. The nature of this activation will be investigated.

The Metabolism of Heterocyclic Compounds

Nicotinic acid degradation. The mechanism by which nicotinic acid is converted to propionate, acetate CO_2 and ammonia has been studied further. Earlier studies showed that 6-hydroxynicotinic acid, 6-oxo-1,4,5,6-tetrahydronicotinic acid and a-methyleneglutarate are intermediates. The hydroxylase that catalyzes the oxidation of nicotinate to 6-hydroxynicotinate has been purified to a state of near homogeneity. The isolated enzyme has a molecular weight of about 300,000 as determined by sedimentation equilibrium measurements, and contains a minimum of 11 moles of iron, 6 moles of labile sulfide and 1.5 moles of FAD per mole. It is therefore a non-heme iron flavoprotein. The enzyme catalyzes the reversible oxidation of nicotinate to 6-hydroxynicotinate when TPN is supplied as the electron acceptor. Nicotinate oxidation can also be coupled with reduction of various dyes including methyl viologen and triphenyltetrazolium hydrochloride. In addition the enzyme catalyzes the oxidation of TPNH by molecular oxygen and exhibits deaphorase activity.

An enzyme catalyzing the conversion of 6-hydroxynicotinate to 6-oxo-1,4,5,6-tetrahydronicotinate was previously purified and shown to require reduced ferredoxin as an electron donor. Further conversion of the 6-oxo-tetrahydro compound to an aldehyde derivative was also repeated. It has now been definitely established that the aldehyde derivative is a-formylglutarate. The next established intermediate in the degradation of nicotinate is a-methyleneglutarate. Although cell-free extracts catalyze the decomposition of a-methyleneglutarate, lability of the products formed has up to now prevented their isolation and identification. Results to date indicate that with partially purified enzyme preparations the substrate is converted to an initial product with which it is in equilibrium; the equilibrium constant for this reaction is about 1.0. When supplemented with small amounts of a relatively crude enzyme preparation the product is converted to a derivative having a carbonyl group, and which can therefore be estimated as the 2,4-dinitrophenylhydrozone derivative. Whereas neither of the products are identified, preliminary data suggests that the formation of one or both involves a vitamin B_{12} dependent reaction.

Anaerobic Energy Metabolism

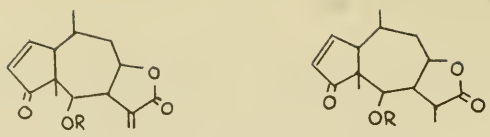
Fatty Acid Kinases. Kinases catalyzing the reversible transfer of the γ-phosphoryl group of ATP to the carboxyl groups of short chain fatty acids, forming the corresponding acyl phosphate derivatives, are widely distributed in microorganisms, where they have an important roles in energy metabolism. In order to determine the mechanism of phosphoryl group transfer at the "energy-rich" level, a study was undertaken to purify the kinases from Clostridium glycolicum. Crude extracts of this organism catalyze the ATP dependent phosphorylation of fatty acid having 2 to 8 carbon atoms. A 60 fold purification of the butyrate kinase activity has been obtained. The presence of more than one kinase in soluble extract is indicated by the fact that with purification the rotio of acetate kinase activity to butyrate kinase activity varies from 5:1 to 0.5:1. Of particular interest was the discovery that both acetate and butyrate kinase activities of the partially purified enzyme are markedly stimulated by carbon dioxide.

Clostridium sticklandii p-Nitrophenylphosphatase. In the course of investigations on the phosphorylation reactions associated with the reductive deamination of amino acids, it was discovered that extracts of C. sticklandii contain a very active, highly specific p-nitrophenylphosphatase. The possibility that this enzyme is normally concerned with electron transport mediated phosphorylation was suggested by its absolute requirement for menadione or a similar quinone derivative and also by the fact that it is specifically activated by AMP or ADP. This enzyme has now been isolated in good yield as a homogeneous protein and studies of its physical properties and mechanism of action have been initiated. Preliminary data suggest that the phosphoryl group of ^{32}P-labeled-p-nitrophenylphosphate is transferred to the enzyme and that the ^{32}P-labeled enzyme can be separated from reactants by gel filtration.

Application of Physical Methods in the Determination of Structures of Organic Compounds

Spectroscopic studies of α-Diketones: Camphorquinone, an optically active α-diketone in which the diketone grouping is held in a rigid conformation, presents itself as a molecule of great theoretical interest for the understanding of the spectroscopic properties of conjugated diketones. Camphorquinone was prepared by the action of selenium dioxide on camphor. Its visible and ultraviolet spectra, optical rotatory dispersion and circular dichroism curves were determined in solvents of varying degrees polarity. Infrared, Raman as well as emission and excitation spectroscopic data were also obtained. As analgous compounds, camphor and 3-methylenecamphor, were studied. 3-Methylene camphor was prepared by base catalyzed consensation of camphor with ethyl formate, followed by reduction with potassium borohydride and elimination of water by alcoholic potassium hydroxide. Consideration of all the experimental data and the theoretical treatment of the molecular orbitals of the conjugated diketone led to the assignment of the 480 mμ-band of camphorquinone to an electronically allowed $n_1 \rightarrow \pi_3$ transition combined with a vibronic component of the $n_2 \rightarrow \pi_3$ transition, while that of the 280 mμ-band to a $n_1 \rightarrow \pi_4$ transition. This study also indicates that the four atoms involved in the conjugated diketone system are at least slightly skewed out-of-plane.

Mass Spectroscopic Study of Sesquiterpenoid Lactones: Continuation of the mass spectroscopic study of sequiterpenoid lactone led to the recognition of characteristic peaks at m/e 95, 96, 122, 123 and 124 for compounds having structural features A and B. High-resolution studies revealed that peaks at m/e 123 and 124 were doublets (C_8O and C_7O_2 fragments) for the A-type compounds and essentially singlets (C_8O fragment) for the B-type compounds. A mechanism for the fragmentation that could accommodate these observations was postulated, and the mass spectra of three different deuterium labeled derivatives substantiated the proposed mechanism

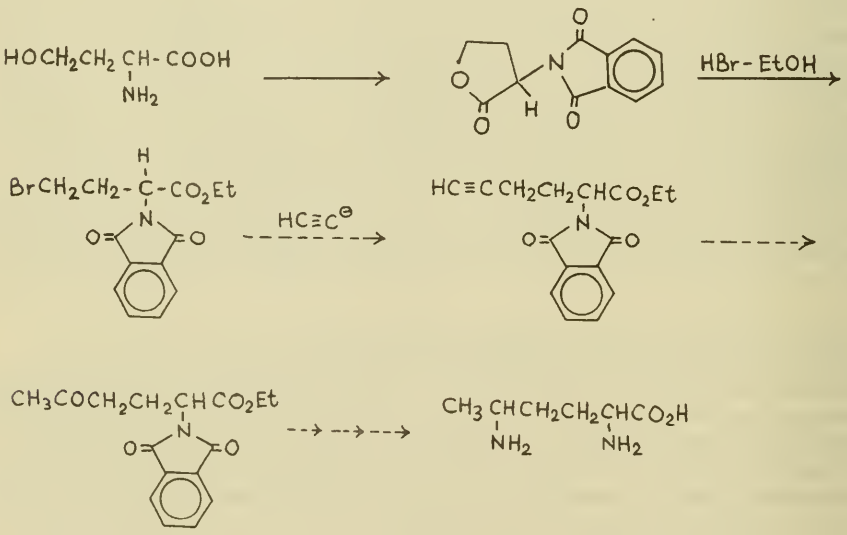


(R = H or Ac)

A B

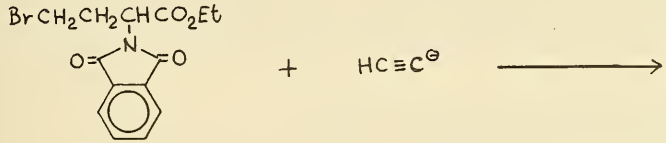
Synthetic Studies of Organic Compounds of Biological Interest

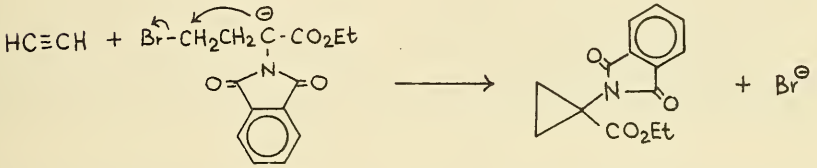
2,5-Diaminohexanoic Acid: Studies on the metabolism of lysine by T.C. Stadtman demonstrated that 2,5-diaminohexanoic acid was one of the intermediates. In order to provide material for further study, a practical synthesis of this compound is much desired. One approach to this problem is to employ homoserine as the starting material for the sequence of reactions outlined below:



This approach offers the advantage of maintaining the stereochemistry of homoserine throughout the steps, thus the final product would possess an α-amino group of known absolute configuration. Unfortunately, the coupling reaction between the bromo

compound and metal acetylide did not proceed in the desired course, instead, a complex mixture was obtained from which a crystalline compound was isolated in pure state. A cyclopropane structure, C, was assigned to this compound on the basis of its NMR, infrared and mass spectra. Its formation could be rationalized as an intramolecular nucleophilic displacement:





C

This rationale was supported by the observation that a much improved yield of the cyclopropane compound was obtained when the bromo compound was treated with sodium ethoxide in anhydrous tetrahydrofuran. Although these experiments failed to achieve the original goal, they have provided a convenient synthesis of a cyclopropane α-aminoacid.

PHS-NIH

Annual Report of the Section on Cellular Physiology
July 1, 1967 through June 30, 1968

As in past years the Section of Cellular Physiology has directed its attention to the following areas of investigation: 1) Structural analysis and biochemical activities of the proteins of the contractile system of muscle; 2) The structure of fibrinogen; 3) Radiation damage in proteins; 4) The mechanism of protein synthesis and its relationship to cell structures; and 5) The biochemistry and cytology of cell transport. These activities are broadly grouped into two areas of biochemistry and cellular physiology; structure and structure function relationships of proteins on the one hand and the role of cell membranes in active transport and synthetic activities of cells.

Proteins of the contractile system of muscle.

Myosin:

A few years ago it was concluded from several lines of evidence that the myosin molecule is constructed from only a single type of subunit polypeptide chain. While it was known that myosin contained traces of low molecular weight protein material these were considered contaminants until recently when it was suggested that they were an integral part of myosin. While the amount of this low molecular weight material in our myosin preparations has been minimal in comparison to published values, during the past year, an extensive examination of the preparative procedure has led to further reduction until at 2.5-5.0% of the mass it appears very improbable that these low molecular weight protein components are actual subunits of myosin.

Previous reports have summarized some limited amino acid sequence data for myosin and last years report dwelt on the use of the cyanogen bromide cleavage procedure for obtaining larger fragments than those provided by proteolytic digestion. 70% formic acid as a solvent allows complete reaction of the methionine residues and fractionation of the peptide mixture is currently proceeding by gel filtration in 5% formic acid with subsequent fractionation of the water soluble fractions by ion exchange chromatography. Dilute HCl on the other hand allows only 70% cleavage and it has been discovered that this is due primarily to the inaccessability of the methionine residues in the light meromyosin (LMM) portion of the myosin molecule. By careful control of the conditions it is now possible to produce LMM which contains no homoserine (the product of the cyanogen bromide reaction) indicating that this type of LMM has no internally broken chains and missing peptide segments (a problem with enzymatically prepared LMM) and confirms our earlier conclusions from amino terminal amino acid analysis that the carboxyl terminus of the polypeptide chains of myosin were at the end of the LMM portion of the myosin molecule.

In the sequential blocking of the two active center sulfhydryl groups of myosin (S_1 and S_1S_2 blocked myosin), it has been assumed that SH blocking interferes with substrate binding and not the catalytic process itself. Binding studies with native myosin, S_1 and S_1S_2 blocked myosin now

completed indicate that all three forms of the protein bind one mole of nucleoside triphosphate (ATP, ITP, GTP, CTP) per mole of subunit with essentially no effect of SH modification on the binding constants. ADP is also bound mole per mole, but none of the other diphosphates are bound. Binding constants are in the order: ATP > ITP > CTP > GTP > ADP.

The apparent conformation change of myosin induced by ATP in the formation of S_1S_2 blocked myosin with N-ethyl maleimide is brought about only by the structurally related nucleotides ATP, ADP and CTP. ITP and GTP either have no effect or inhibit the blocking depending on whether the major cation is K^+ or Na^+ respectively. However, all of the nucleotides appear to produce some structural change when bound which makes available specific peptide bonds for proteolytic attack for all of the nucleotides that are bound double or triple the rate of inactivation of myosin by trypsin or chymotrypsin.

Actin:

It is now quite well established as a consequence of previous work in this laboratory on the preparation of pure G-actin and on its molecular parameters, that actin has a molecular weight of 47000, consists of a single polypeptide chain and possesses one mole of the unusual amino acid, 3-methyl histidine, per mole of protein. Activities during the past year have been directed toward application of the cyanogen bromide cleavage reaction to actin and separation of the resulting fragments. Of the sixteen anticipated peptide fragments ten have now been isolated in pure form, including the one containing the 3-methyl histidine residue, and are currently being subjected to some limited amino acid sequence studies. Human platelets possess an actomyosin like protein from which a crude actin-like component has been obtained. This material possesses 3-methyl histidine in about the same ratio to histidine as in muscle actin.

Structure of fibrinogen:

Alkali denaturation of fibrinogen causes an increase in specific optical rotation, an increase in UV absorption in the region of the phenoxide ion absorption, an increase in the fluorescence of a dimethylaminonaphthalene sulfone group coupled to the protein and a loss of solubility at neutral pH. Kinetic studies show that the first three changes are simultaneous whereas the loss of solubility occurs at a much faster rate. All these reactions are of the first order type and there is no evidence of more than one reaction class in the overall process. The results have been rationalized on the basis of the three bead model of the molecule. If the three beads unfold independently of each other but at the same rate there will be only a single reaction and the optical changes will reflect the average unfolding of the whole molecule. On the other hand, if unfolding of a single bead in a molecule is sufficient to cause loss of solubility at neutral pH, then the latter reaction will occur at a much faster rate. Fitting curves, based on statistical distribution of molecules with none, one, two or three beads unfolded versus time, to the experimental curves of average fraction unfolded and fraction insoluble, the best fit is obtained with the assumption that unfolding of a single bead is sufficient to cause insolubility.

In previous work it was observed that brief tryptic digestion of fibrinogen

(molecular weight 350,000) led to the formation of physically homogeneous material of molecular weight about 90,000. These fragments have now been separated into three distinct species. However, these three fragments have fairly similar chemical properties suggesting a high degree of symmetry within the parent molecule. On the other hand when the disulfide bonds of fibrinogen were cleaved and the resulting material subjected to ion exchange chromatography, four components were obtained with a molecular weight range of 45,000-60,000. From end group analysis, it has been suggested by other investigators that there are three pairs of three subunits in fibrinogen. Of the four components isolated in the present effort the first two peaks from chromatography appear to be very similar based on molecular weight, amino acid analysis and fingerprints. It seems possible that they are the same component with one being the consequence of slight modification during disulfide cleavage reaction. While this is still under investigation it appears that the three subunits are now available for further analysis and comparison with the tryptic fragments from which it will be possible to determine how the subunits are arranged in fibrinogen.

Radiation damage in proteins:
The method of radioactive labeling of carbon free-radicals resulting from gamma irradiation using tritium donors (e.g. tritiated hydrogen sulfide, tritium iodide) has been further investigated with respect to mechanism. From the use of tritium donors involving different reaction mechanisms it has been concluded that the distribution of tritium is equivalent to the distribution of free radicals prior to exposure to the tritium donor.

In the gamma-irradiation of proteins, it appears there is a direct primary distribution of free radicals which rapidly exchange to give the more stable secondary free-radical distribution which reacts with the tritiated free radical interceptor. This has been tested by conducting irradiation and tritiation at liquid nitrogen temperatures. The free radical distribution obtained under these circumstances was similar to that obtained for all denatured proteins at room temperature, suggesting that the characteristic free radical distribution of ntaive proteins irradiated at room temperature is a function of the influence of tertiary structure on secondary free radical formation.

The free-radical interceptor technique has also been explored as a general method for radioactive labeling of proteins. As such it would have some virtures over introduction of foreign groups which might have subtle effects on protein conformation. As a beginning it has been demonstrated that tritiation does not alter the physical or enzymatic properties of either ribonuclease or lysozyme. Particular attention was paid to the possibility that free radical formation might lead to racemization of the gamma-carbon atom of the affected amino acid residues. It was demonstrated that this does not occur.

Protein synthesis and its relationship to cell structure:
Procedures for preparing and isolating membrane fragments from E. coli spheroplasts with the least amount of physical damage have been developed. Electron microscopy of this material shows the larger membrane fragments as either open ovals or spirals, the smaller fragments appearing mostly as

closed vesicles. Electron dense granules with the size and appearance of ribosomes line the inner surface of most of the fragments. Chemical analysis indicates that these membrane preparations are relatively rich in RNA when compared to literature values for preparations made by more drastic procedures. Analysis of two types of fractions differing in their whole cell contamination indicates that the slight contamination while contributing to the protein synthetic ability of this preparation is not responsible for the bulk of the activity. Comparison of this synthetic ability with published values for fully primed E. coli ribosomal systems stimulated with messenger RNA indicates that these membrane preparation appear to be operating at several fold greater efficiency without addition of exogenous messenger.

In parallel investigations aimed at relating electron transport energy pathways in the membrane to protein synthesis, the distribution of dehydrogenase and oxidase activities of the various fractions obtain in membrane fragment isolation has been examined. There is a very active membrane bound NADH-oxidase system, sensitive to cyanide but, in contrast to conventional mitochondrial systems, it is insensitive to other inhibitors of electron transport, activators or inhibitors of coupled phosphorylation or uncoupling agents. Soluble malate and isocitrate dehydrogenases are present requiring NAD and NADP respectively. However oxidation of malate and isocitrate in this system is not efficiently linked to oxygen uptake and there is a suggestion that an intact Krebs cycle system may not be present.

The biochemistry and cytology of transport:
Morphological studies of Acanthamoeba have been continued, the studies covering active growth through encystment. The amoeboid form has several interesting features including microtubules and microfibrils, the latter are especially prominent in pseudopods and in the hyaline layer that underlies the plasma membrane. These structures may be of importance in the movements associated with phagocytosis. In this regard, attempts to isolate an actomyosin-like system using the procedures employed in obtaining such a protein system from slime mold have been unsuccessful so far.

The morphology of the contractile vacuole, the organelle responsible for water and ion secretion has been carefully studied. The fine structure of the cyst wall has been described and the ultrastructural changes leading to its formation have been delineated. There are major changes in the mitochondria, endoplasmic reticulum and Golgi membranes. The latter swell with dense material that is transported through the plasma membrane and deposited as cyst wall. The morphological changes have been correlated with a decrease in glycogen, a decrease in phospholipids and an increase in glycerides. Another obvious change is the appearance of autolysosomes which engulf cytoplasmic constituents and degrade them with autolytic enzymes. This probably represents the first reasonably complete study of the ultrastructure of an amoeba.

Phagocytic vesicles have been isolated in essentially quantitative yield. Two fractions are obtained; the lighter one represents newly formed vesicles and these contain very low concentrations of enzyme. The

heavier fraction represents digestive vacuoles with a much higher content of hydrolytic enzymes.

The lipid composition of the whole cell and of the phagocytic vesicles has been defined, at least with respect to the major components. Whiel the major phospholipids of the cell are phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine in decreasing order, in the membranes the relative amounts of the choline and ethanolamine phospholipids are reversed and there is somewhat more phosphatidyl serine than in the whole cell. Both lipid fractions contain material that behaves like cardiolipin. Some lipophosphatidyl choline and material that may be glyco and sulfolipids is also present. The sterol content of the vesicle lipids is 2-3 times higher than the lipid of whole cells. Two sterols are present, ergosterol and 7-dehydrostigmasterol. The latter has not been demonstrated in protozoa previously. Examining the incorporation of radioactive phosphate into phospholipids of the whole cell and phagocytic vesicles, no effect of phagocytosis on membrane turnover or synthesis can be demonstrated.

Serial No. NHI- 1
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Mechanism of Action of Ethanolamine Deaminase

Previous serial number: 137

Principal Investigator Bernard M. Babior

Other Investigators: E.R.Stadtman

Cooperating Units: None

Man Years:

Total:	1.3
Professional:	1.0
Other	0.3

Project Description:

Objectives: To study the mechanism of action of ethanolamine deaminase with emphasis on the role of coenzyme B_{12}.

Major Findings

Experiments with tritiated substrate have shown that the transfer of hydrogen is stereo-specific with respect to which of the two H-atoms on the alcohol carbon are transferred. The oxygen of acetaldehyde was found to be derived from the substrate, rather than from water.

The results reported previously that certain B_{12} analogs could replace coenzyme were found to be due to contaminating coenzyme. These analogs, as well as a number of others, were found to inhibit the reaction. However, the B_{12} analog-induced tight binding of ethanolamine was confirmed. A study of inhibitor kinetics led to the conclusion that the enzyme exists in two forms, which vary in their V_M and in their affinity for various inhibitors. In the presence of an inhibitor the enzyme changes from one form to the other.

27

Binding of coenzyme and of hydroxy B_{12} to the enzyme leads to a change in the circular dichroism of the cobalamines. Titrations by this technique showed that the enzyme possessed 2 binding sites for these compounds. Kinetic experiments confirmed this result and in addition showed that each binding site represented a separate and independent active site.

Aerobic photolysis of alkyl cobamides leads to the production of hydroxy B_{12} via the homolytic cleavage of the carbon-cobalt bond followed by oxidation of the resulting B_{12_Y}. Preliminary studies with a number of enzyme-cobamide complexes have shown that aerobic photolysis leads to products other than hydroxy B_{12}. In particular, ESR studies indicate that the enzyme is capable of stabilizing a paramagnetic species produced by the photolysis of both methyl B_{12} and coenzyme. There is preliminary evidence that in the presence of acetaldehyde, the enzyme is capable of splitting the coenzyme at the carbon-cobalt bond.

Course of Action:

Continuation of studies of the effect of photolysis on enzyme B_{12} complexes. Further study of the splitting of coenzyme by enzyme in the presence of acetaldehyde.

Publications

1. Barry H. Kaplan and E.R. Stadtman, Ethanolamine Deaminase, A Cobamide Coenzyme-Dependent Enzyme II. Physical and Chemical Properties and Interaction with Cobamides and Ethanolamine, J.Biol.Chem. 243, 1787 (1968).

2. Barry H, Kaplan and E.R. Stadtman, Ethanolamine Deaminase, a Cobamide Coenzyme-dependent Enzyme I. Purification, Assay, and Properties of the Enzyme, J. Biol. Chem. 243, pp 1787-1793 (1968).

Serial No. NHI- 2
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHI
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Metabolism of Heterocyclic Compounds

Previous Serial Number: 135

Principal Investigator: Stephan Cederbaum

Other Investigators: E.R. Stadtman

Cooperating Units: None

Man Years:

total:	1.4
Professional:	1.1
Other	0.3

Project Description:

Objective: To study the steps in the conversion of α-methylene glutaric acid, an intermediate in the metabolism of nicotinic acid by a clostridial species, to acetate, propionate and carbon dioxide.

Major Findings:

Efforts to purify the enzyme(s) involved led to loss of activity that could not be restored by alkaline carbonate treatment. However, activity could be restored by pre-incubation of the inactive enzyme preparation with a crude extract from C. sticklandii. Activity of the C. sticklandii extract was shown to be due to a heat labile, large molecular weight component, and a heat stable factor that can be replaced by FAD. Alkaline carbonate remained a mandatory part of the reactivation system. The relationship between the elements involved in the reactivation remains to be elucidated. Making use of the reactivation, the crude extract has been purified 15 fold, with excellent recovery of activity. The apparent equillibrium constant remains about one. No cofactor requirements are demonstrable for this reaction. The need for iron and glutathione in the routine assay may be spared if the reaction mixture is rendered anaerobic prior to the addition of the enzyme preparation.

The products of α-methylene glutaric decomposition are still unidentified despite numerous efforts. Identification has been hampered by the extreme instability of the product(s) to mild isolation procedures. It has been possible to make a stable trimethylsilyl derivative of one of the products (product I). On the basis of its behavior on a gas-liquid chromatography it was concluded that there are three trimethylsilyl groups per molecule. Combined with titration data it seems probable that there are two carboxyl groups and a third oxygen function capable of forming an ether with the trimethylsilyl moiety. A second labile product (II) has been shown to accumulate when substrate is incubated with crude extracts. This product has been partially purified as its 2,4-dinitrophenylhydrazine derivative. The yield of this product may be increased by the addition of a suitable hydrogen acceptor, FMN serving this function best.

To determine if the stimulation by bicarbonate involves a CO_2-fixation reaction, experiments with $^{14}CO_2$ were carried out. Incubation of α-methyleneglutarate and $^{14}CO_2$ with crude extract resulted in incorporation of labeled carbon into the residual α-methyleneglutarate and into both products I and II. The CO_4-fixation reactions are not inhibited by avidin.

Proposed Course of Project:

1) Continue efforts to purify the first enzyme in the sequence; 2) Elucidation of the nature of the alkaline carbonate-Cl. sticklandii reactivation: 3) Identification of the 2,4-DNP derivative formed and study of the reaction responsible for its formation; 4) Study the CO_2 fixation reaction.

Publications: None

Serial No. NHI-3
Laboratory of Biochemistry
Section on Enzymes
PHS-NIH Bethesda, Maryland

Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Enzyme Structure and Mechanisms of Action and Control

Previous Serial Number: 132

Principal Investigator: D.Denton, M.D.

Other Investigators: Ann Ginsburg, Ph.D.

Cooperating Units: none

Man Years:

Total:	1.4
Professional:	1.1
Other:	0.3

Project Discription:

Objective: 1) To correlate the structure and catalytic activity of glutamine synthetase from E. coli with physical, chemical and enzymatic studies.
2) To study the specific binding of small molecules to various preparations of glutamine synthetase and to correlate the relationships of the binding of effectors to enzyme catalyses and regulation.
3) To purify the ATP-glutamine synthetase adenylyltransferase from E. coli.

Major Findings

Direct binding studies of manganese^{54} to two different native preparations of glutamine synthetase which vary in their degree of adenylylation have been performed. Extended studies indicate that there are 3 classes of Mn^{+2} binding sites as reported in the last annual report. However there is one very tight Mn^{+2} binding site per subunit and not one per covalently bound AMP as previously reported. Varying degrees of adenylylation however do affect the apparent K'_A of the binding. The less adenylylated glutamine synthetase has a much higher affinity for Mn^{+2} at the tighter site whereas the affinities of all other sites are influenced by adenylylation. The results are consistent with theinterpretation that there are 12 very tight sites (one per subunit) at which the binding of Mn^{+2} is accompanied by conformational changes in the protein. The ΔF of this conformational change is a function of the average state of adenylylation of the enzymes. There are 12 other sites of intermediate affinity that together with the very tight sites are probably concerned with the catalytic activity. Finally, there are a large number (up to 48) of lower affinity sites that

appear to be associated with gross stabilization of the enzyme.

Mg^{+2} and Ba^{+2} competition with Mn^{+2} binding to glutamine synthetase has been studied also. The competition of Mg^{+2} with the highest affinity sites for Mn^{+2} give $K'_A = 4 \times 10^5$ for the $E_{\overline{2\,3}}$ preparation at pH 8, as compared to $K'_A = 1.5 \times 10^7$ for Mn^{+2} under the same conditions. Ba^{+2} has $K'_A = 200$ which is consistent with the non-specificity of this ion. Mg^{+2}, but not Ba^{+2}, is effective catalytically or physically in inducing the conformational change associated with the relaxed to taut conversion. On this basis, the overall free energy change for the Mg^{+2} conversion must be lower than that for Mn^{+2}

Equilibrium binding and kinetic studies of the ATP-enzyme interaction indicate that there is a relationship between the number of covalently attached AMP groups and the degree of cooperativity observed in the binding of 12 equivalents of the substrate ATP. ATP binding is linked to the divalent cation binding and the interaction is negative.

The ATP-glutamine synthetase adenylyltransferase has been purified 60-100 fold and studies on the mechanism of action are in progress.

Proposed Course of Action

The above studies will be extended during the remaining period covered by this report. June 30, 1968 will complete my tour of duty with the USPHS and separation from active service is planned.

Publications None

Serial No. NHI- 4
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHI
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Purification and Properties of Bacillus subtilis Glutamine Synthetase

Previous serial No.: None

Principal Investigator: Thomas F. Deuel

Other Investigators: None

Cooperating Units; none

Man Years:

Total	1.5
Professional:	1.2
Other:	0.3

Project Description:

Objectives: Detailed investigations from this laboratory have demonstrated in E. coli a unique form of cumulative end-product feedback inhibition by a variety of products of glutamine metabolism. Kinetics and physical chemical studies of the purified enzyme have further clarified many structural features associated with catalytic activity, and established enzymatic modification of the glutamine synthetase molecule as a unique mechanism modifying greatly metal ion specificity and inhibitor response. Other organisms surveyed have demonstrated end product inhibition largely specific for the organism studied. In view of these findings, the present study was initiated to provide detailed information on glutamine synthetase from an unrelated microorganism, with the ultimate aim of providing structural and functional correlates for the complex interactions of glutamine synthetase.

Major Findings:

High specific activities of glutamine synthetase are found in crude extracts of B. subtilis 10029, an alanine dehydrogenase negative tryptophan requiring mutant. A 3-5 fold increase in specific activity is obtainable utilizing glutamate as opposed to ammonia as nitrogen source for growth in a minimal salt medium, although growth rate is retarded by a factor of 2 in glutamate growing cells. Utilizing streptomycin precipitation, ammonium sulfate fractionation, heat and acid fractionation in the

presence of ATP and glutamate, and agarose gel filtration, a 25-30 fold purification has been achieved. Six bands are present on polyacrylamide gel electrophoresis, resolving to a single band after reduction of the enzyme with 10^{-2} M Clelands reagent, treatment with 10^{-2} M EDTA, and M urea. Two peaks are present in ultracentrifuge studies, again resolving to a single peak if sedimented in the presence of 10^{-2} M Clelands reagent, 10^{-2} M EDTA, and 7 M urea. S_{w20} for the faster sedimenting peak is 18.8, and for the slower peak 14.1, with or without Clelands reagent present. The reduced urea dissociated preparation has a S_{w20} of 1.33 in 7 M urea.

The enzyme is most active as measured by a biosynthetic assay measuring glutamate dependent P_i release from ATP. Manganese appears required for the reaction, although with less effectiveness magnesium may serve as a divalent cation source. Preliminary evidence suggests that while either Mg ATP or Mn ATP may serve as substrate, a separate Mn^{++} site is present required for maximum catalytic activity of the enzyme. Clelands reagent or mercaptoethanol enhances activity by a factor of 250%. High ionic strength enhances catalytic activity; preliminary evidence indicates the effect is secondary to enhanced stability of the enzyme, shown at temperatures above 30., and most markedly seen with Mg^{++} as the divalent cation present in the assay.

Proposed Course of Project:

The nature of the two peaks seen in ultracentrufuge patterns will be explored. Detailed kinetic structure of the enzymatic reaction are planned, and the interactions of inhibitors and substrates investigated. Further physical chemical characterization of the protein will be done.

Publications: none

Serial No. NHI-5
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Enzyme Structure and Mechanisms of Action and Control

Previous Serial Number: NHI 132

Principal Investigator: Ann Ginsburg

Other Investigators: Dr. M.D. Denton
Dr. B. M. Shapiro

Cooperating Units: none

Man Years

Total:	2.5
Professional:	1.2
Other:	1.3

Project Description:

Objectives: 1) To study the physical and chemical properties of glutamine synthetase from E. coli, particularly with respect to the correlation of the structure and catalytic function of this enzyme. 2) To study conformation and stabilization changes of a protein macromolecule effected through the specific binding of small molecules, and the relationship of such effects to enzyme catalysis and regulation. 3) to purify and study the ATP-glutamine synthetase adenylyl transferase from E. coli with emphasis on the mechanism of action of this enzyme.

Major Findings:

Studies on the equilibrium binding of the feedback inhibitor adenosine 5'-phosphate (AMP) to glutamine synthetase have been extended. The binding of AMP to three different preparations of glutamine synthetase which vary chemically only in the extent of adenylylation (covalently bound AMP) has been found to be the same. (This must be correlated to the observations of Kingdon, Shapiro, and Stadtman that these enzyme forms (denoted as $E_{o,1,2}$---12AMP) differ dramatically in their divalent cation specificity and response to feedback inhibitors during catalysis). The binding characteristics of AMP to glutamine synthetase preparations (in the presence of M/10 KCl and imidazole buffer at pH 7.4-7.5) show, a) there are 12 independent and

equivalent binding sites per 600,000 g of protein or one per subunit; b) the apparent association constant (k'_A) is about 8000; c) the binding of AMP is predominently of a hydrophobic rather than of an electrostatic nature; d) AMP binding is not influenced by Mn^{2+}, Mg^{2+}, or the presence of EDTA (which produces a relaxed inactive enzyme form.) When [^{14}C] - or [^{32}P] - AMP was used in conjunction with non-isotopically labeled compounds, the data suggest a) GMP, adenosine, and adenine (not UMP, CMP or IMP) competitively bind at the AMP sites where k'_A for adenosine > adenine >> GMP; b) the substrate(s) glutamate and/or ammonia enhance AMP binding. The equilibrium effect binding of L- [^{14}C] tryptophan to glutamine synthetase shows a) the binding is independent of the extent of adenylylation of the preparation; b) the binding is the same with Mn^{2+} or Mg^{2+} present; c) there is cooperativity in the binding of tryptophan with a Hill coefficient of about 2.5 and about 700 cal. estimated for the free energy of interaction· d) half saturation is at 0.9 mM L Tryptophan; e) at saturating tryptophan concentrations 12 equivalents of tyrptophan are bound. [^{14}C] Tryptophan binding in the presence of [^{32}P] AMP has indicated: a) tryptophan binding is not effected by the presence of AMP; b) AMP binding is decreased by tryptophan binding and this effect is enhanced by the presence of 1 mM Mg^{2+} rather than 1 mM Mn^{2+}; c) Higher concentrations of tryptophan (beyond the conformational transition) decrease the amount of AMP bound more than do low levels of tryptophan. L-[^{14}C] alanine binding parameters could only be approximated since this inhibitor has a low affinity for the enzyme and further more is not bound at all in the absence of substrate (glutamine). However, the binding of [^{32}P] AMP was unaffected by [^{14}C] alanine plus glutamine, and conversely, AMP did not effect the binding of alanine. The binding of L -alanine is cooperative. The equilibrium binding data suggest that tryptophan is partially competitive with substrate (glutamate) kinetically through an antagonistic action stabilizing different conformations. AMP must exert its kinetic effects only when coupled with substrate and metal. (Manuscript by A. Ginsburg in preparation).

The equilibrium binding data of Dr. M.D. Denton, which are complementary to these and other studies (below), show: a) the binding of the substrate ATP also occurs to the extent of one equivalent per subunit but in this case there is a correlation between the state of adenylylation of the glutamine synthetase molecule and the ATP-Mn^{2+} binding: b) the most specific Mn^{2+} binding is associated with a conformational change in the glutamine synthetase molecule which is a function also of the average number of covalently bound AMP molecules. This metal binding correlates to the stabilization by Mn^{2+} of the active (taut) form of the enzyme (divalent cation-free (relaxed enzyme is inactive). Approximately 12 Mn^{2+} are associated with the conformational change and these, together with a 2nd class of 12 sites of slightly lower affinity, probably have a catalytic function. A large number of less specific Mn^{2+} binding sites appear to be associated with a gross stabilization of the protein. (Manuscript by D.M. Denton and A. Ginsburg in preparation.)

The physical and chemical changes induced by Mn^{2+} (Taut versus relaxed enzyme forms) have been investigated with Dr. B.M. Shapiro. Three different preparations of glutamine synthetase were investigated and found not to differ significantly with respect to the taut-relaxed conversion as judged by sulfhydryl reactivity, reactivation at saturating substrate concentrations (normalizing specific activities in a Mg^{2+} assay system) or difference spectra where tyrosine and tryptophan residues are exposed upon metal removal. (The k_D^1 of the induced difference spectra correlates with the highest affinity $^{54}Mn^{2+}$ binding). Relaxed enzyme forms of different states of adenylylation had the same hydrodynamic properties (sedimentation, viscosity and apparent specific volume). However, subtle differences between these properties of the taut forms in varying states of adenylylation were detected. This observation again correlates with the $^{54}Mn^{2+}$ binding data. (Manuscript by B.M. Shapiro and A. Ginsburg accepted by Biochemistry for publication).

The studies on the ATP:glutamine synthetase adenylyltransferase (AT) from E. coli are in collaboration with Dr. M.D. Denton. It appears that the reaction catalyzed by this enzyme is the sum of two half-reactions.

1) $ATP + AT \underset{}{\overset{Mg^{2+} \text{ or } Mn^{2+}}{\rightleftharpoons}} AT\text{-}AMP + P\text{-}P_i$

2) $AT\text{-}AMP + \text{glutamine synthetase } (E_o) \rightleftharpoons E_1\text{---}12AMP + AT$

$\overline{Mg^{2+}}$

Reaction 1) is catalyzed in the presence of high concentrations of glutamate or glutamine and either Mg^{2+} or Mn^{2+}. The adenylylation of glutamine synthetase (reactions 1 and 2) is inhibited by glutamate, α-ketoglutarate, RNA, t-RNA, CTP UTP, GTP, CDP, UDP, GDP, ADP, organic mercurial reagents, PP_i, P_i and Mn^{2+} (the effect of Mn^{2+} is on the substrate glutamine synthetase in that it makes the latter less susceptible to adenylylation); the Km for ATP is 1.3 mM, the Mg^{2+} form of glutamine synthetase is saturating at ~5 mg/ml, glutamine which is required in (2) is saturating at 1 mM and sulfhydryl compounds stimulate the adenylylation reaction. Reaction (2) appears to be specific for glutamine synthetase; bovine serum albumin, lactic acid dehydrogenase, or N-acetyl L-tyrosinamide do not act as substrates for the adenylyltransferase. Glutamine synthetase plus glutamine inhibit reaction (1) and GTP, UTP, CTP or ITP will not substitute for ATP in (1).

The adenylyltransferase is quite stable and has been purified 70-100 fold by Dr. M.D. Denton.

Significance to Biomedical Research: The regulation and control of enzymic activities in vivo is of fundamental importance in cellular metabolism. Through in vitro studies on a molecular level these processes can be more fully understood.

37

Proposed Course of Action:

Some of the physical properties of homogeneous preparations of non- and fully adenylylated glutamine synthetase will be investigated. Dissociation of the adenylylated enzyme to its constitutive subunits under mild conditions will be followed by studies of the binding of AMP to the subunits to access the role of the quarternary enzyme structure in the binding of effectors. The effects of substrate binding on inhibitor binding to the native enzyme also require further investigation. Physical studies of the adenylylated glutamine synthetase in 6 M guanidine-HCl will be made to reveal whether the previously found subunit of 50,000 molecular weight is composed of one or two polypeptide chains. This investigation is prompted by considerations of substrate and effector binding to the macromolecule. Carboxypeptidase digestions of glutamine synthetase will be studied in conjunction with the dissociation. Carboxypeptidase digestion of rabbit muscle aldolase also is planned to find out if the small amount of organic phosphate in native enzyme preparations is located near the carboxy terminal end. If so, the binding of a substrate analog to carboxypeptidase-treated aldolase will be studied to learn if the phosphate on the molecule blocks an equivalent proportion of the catalytically active sites.

The ATP glutamine synthetase adenylyltransferase from E. coli will be purified and studied further, with emphasis placed on learning more about the interaction between this protein and the glutamine synthetase molecule.

Publications

1. Bennett M. Shapiro and Ann Ginsburg, Effects of Specific Divalent Cations on Some Physical and Chemical Properties of Glutamine Synthetase from Escherichia coli: Taut and Relaxed Enzyme Forms, Biochemistry, 1968, in press.

Serial No. NHI- 6
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Role of B_{12}-coenzyme in the Metabolism of Lysine.

Previous Serial Number: 138

Principal Investigator: Margaret Grant

Other Investigator: T.C. Stadtman

Cooperating Units: None

Man Years:

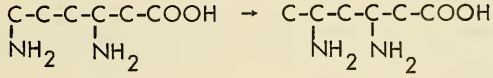
Total:	1.2
Professional:	0.9
Other:	0.3

Project Description:

Objectives: Elucidation of the mechanism of the B_{12}-coenzyme dependent migration of an amino group from carbon 6 of β-lysine (3,6-diaminohexanoate) to carbon 5, to form 3,5-diaminohexanoate - a new amino acid. This reaction is involved in the overall pathway of fermentation of lysine to ammonia and fatty acids.

Major Findings:

The enzyme system which catalyses the conversion of β-lysine to 3,5-diaminohexanoate (Reaction 1) has been called, tentatively, β-lysine mutase. The following



(1)

data concerning this reaction were included in last year's report. First, β-lysine mutase consists of two proteins. One of these is an orange cobamide protein; the other is a sulphydryl protein which will be referred to hereafter as enzyme 2. Secondly, β-lysine mutase requires for activity, a mercaptan, a divalent metal ion

such as Mn^{2+} or Mg^{2+} and cobamide coenzyme. Additional requirements for the reaction are ATP, pyruvate and FAD, which can be replaced, to varying extents, by 2'-deoxy-ATP, α-ketobutyrate and FMN respectively.

An attempt has been made to purify the orange cobamide protein by chromatography on DEAE-cellulose and preparative acrylamide gel electrophoresis. A small amount of this protein (cal mg) has been obtained essentially pure, as evidenced by the presence of only two trace contaminants on analytical disc gels. The purification procedure has been modified to allow larger scale recovery of the protein. This has yielded 6 mg of highly purified material.

Since the cofactors required for the β-lysine mutase reaction include those known to participate in the biosynthesis of B_{12}-coenzyme from vitamin B_{12} and ATP, the possibility that the role of enzyme 2, at least in part, might be to synthesize B_{12}-coenzyme was investigated. Of several crude preparations of enzyme 2 so far examined all exhibit coenzyme-B_{12} synthetase activity. Parallel assays for enzyme 2 activity and coenzyme synthetizing activity are currently in progress as each successive purification step is developed.

The time course of the reaction catalysed by β-lysine mutase is not linear, but exhibits an initial lag period. Since knowledge of the causes of this lag could give insight into the mechanism of the reaction, attempts have been made to eliminate it. These have been unsuccessful. However, it has been found that increasing levels of the coenzyme can reduce the lag markedly.

Using crude preparations of the cobamide protein and enzyme 2, an investigation into the effect on the reaction of increasing amounts of one with fixed amounts of the other has been carried out. The results show that high levels of the cobamide protein preparation do not inhibit the reaction, whereas slight inhibition occurred with high levels of the enzyme 2 preparation.

Proposed Course of Project:

1. To continue the purification and characterization of the two proteins of the β-lysine mutase system.
2. To investigate further the possible role in the reaction of enzyme 2 as B_{12}-coenzyme synthesising system. An attempt will be made to replace enzyme 2 with a preparation, from another organism, which has good coenzyme synthesising activity but which lacks β-lysine mutase.
3. To attempt to eliminate the initial reaction lag.

Publications: None

Serial No. NHI- 7
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Purification and Properties of Salmonella β-Cystathionase.

Previous Serial No. None

Principal Investigator: Stephen Guggenheim

Other Investigator: Martin Flavin

Cooperating Unit: Lowell Owens, U.S.Soils Laboratory, Soil and Water, Conservation Research Division, U.S. Department of Agriculture, Beltsville, Md.

Man Years:

Total:	0.3
Professional:	0.1
Technical:	0.2

Project Description:

Objectives. Purification of β-cystathionase from Salmonella was undertaken because of its possible usefulness in assaying for cystathionine, and also because it was thought to be the enzyme inhibited by rhizobitoxine, a toxin of bacterial origin which causes chlorosis in plants.

Major Findings:

Large quantities of β-cystathionase are easily obtained from an otherwise discarded ammonium sulfate fraction in the purification of cystathionine γ-synthetase. Using DEAE column chromatography, a second ammonium sulfate fractionation, and Sephadex G-200 gel filtration, β-cystathionase can be purified 300 fold from extracts of derepressed Salmonella Me A 15.

The enzyme catalyzes the reaction:

$$\text{Cystathionine} + H_2O \rightarrow \text{Pyruvate} + \text{Homocysteine} + NH_3$$

41

The apparent K_m for cystathionine is 3×10^{-4} M. Cystine is also a good substrate, but cysteine is a poor one. There is a moderately sharp pH optimum at pH 8.5.

Cystathionine is assayed with the enzyme as follows. Aliquots of unknown are treated with dithiothreitol and iodoacetic acid in order to convert cystine to a poorly reactive form. Lactic dehydrogenase and DPNH are added and the change in absorbancy at 340 mμ upon addition of excess β-cystathionase is measured in a spectrophotometer. The method has been successfully used in determining the specific activity of tritiated cystathionine.

As shown by Owens and Wright, (Plant Physiol., 40, (1065), 927), rhizobitoxine is a toxin produced by the bacteria Rhizobium japonicum which causes chlorosis in plants and also inhibits the growth of Salmonella. Although its exact structure is not known it appears to resemble cystathionine in several respects. Together with Lowell Owens we have shown that this is a potent inhibitor of β-cystathionase. Kinetic experiments showed a mixed competitive-noncompetitive inhibition pattern and gave an apparent K_i of 2×10^{-8} M.

Proposed Course of Action

Further purification of β-cystathionase is planned in order to permit studies of the mechanism of rhizobitoxin's interaction with the enzyme. We hope, also to extend the enzymes usefulness in assaying for cystathionine.

Publications

1. L.D.Owens, S.J. Guggenheim, and J.L. Hilton, Rhizobium-synthesized phytotoxin: An inhibitor of β-cystathionase in Salmonella typhimurium. Biochim. et Biophys. Acta (in press).

Serial No. NHI-8
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The mechanisms of γ-elimination and γ-replacement reactions potentiated by cystathionine γ-synthetase from Salmonella typhimurium.

Previous Serial Number: NHI-218

Principle Investigator: Stephen Guggenheim

Other Investigator: Martin Flavin

Cooperating Units: None

Man Years:

Total:	1.3
Professional:	1.1
Technical :	.2

Project Description:

Objectives: The ability of cystathionine γ-synthetase to catalyze reactions in which covalent bonds to the γ-carbon atom of certain amino acids are formed and broken makes it unique among pyridoxal phosphate enzymes. The aim of this project is to investigate the mechanism of the enzyme action by studying the organic chemistry of the reactions catalyzed by the enzyme and the changes in the enzyme's physical properties during the course of the reaction. The role of pyridoxal phosphate in these processes are of special interest.

Major Findings: The γ-elimination reaction with the substrate O-succinylhomoserine

$$\text{O-succinylhomoserine} + H_2O \rightarrow \text{succinate} + \alpha\text{-ketobutyrate} + NH_3 \quad (1)$$

(reaction 1) was carried out in deuterium water to determine whether hydride transfer or proton labilization generates the unsatuated intermediate which must be formed in the γ-elimination reaction. The resulting α-ketobutyrate, isolated as a dinitrophenylhydrazone and assayed for deuterium by nuclear magnetic resonance spectroscopy, contained 1.0 atoms per mole excess deuterium in the β position and 0.2 in the γ position. Although this result did not rule out hydride transfer completely, it more likely indicated a protolytic mechanism in which the enzyme shielded a labilized proton from exchange with solvent water. The deuterated α-ketobutyrate also was degraded to optically active sodium propionate, indicating that addition of a proton to the β-position of an unsaturated intermediate, a relatively late step, was enzymatic.

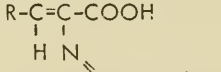
43

Cystathionine, usually an enzyme product in the γ-replacement

O-succinylhomoserine + L-cysteine → L-cystathionine + succinate 2

reaction (reaction 2), is also a substrate for several enzyme catalyzed reactions. It undergoes both β- and γ-elimination. It is a substrate for ^{35}S-cysteine- cystathionine exchange, a type of γ-replacement reaction. Finally, the enzyme catalyzes exchange of the α-hydrogen and one stereospecific β-hydrogen in each half of the cystathionine molecule with solvent hydrogens in experiments carried out in tritiated water. The finding that the exchange of tritium into the 4 carbon chain of cystathianine was 500 times faster than the cysteine- cystathionine exchange reaction indicated that the mechanism of γ elimination was unconcerted, labilization of the β-hydrogen preceding removal of cysteine, the γ-substituent. This interpretation is confirmed by the finding that the enzyme catalyzes hydrogen exchange between water and α-aminobutyrate, an amino acid with no γ-substituent.

The enzyme also catalyzes tritium exchange between water and α- and β- positions of three carbon amino acids such as serine, cysteine and alanine. Although the enzyme catalyzes β-elimination from succinyl serine and cystathionine, in this case the β-hydrogen exchange must be a side reaction rather than part of the β-elimination mechanism. The resulting dead end intermediate, might account for a transient 465 mμ absorption band seen



when the enzyme is saturated with succinylserine and also for the slowing of β-elimination from this substrate which occurs during the first 3 or 4 minutes of the reaction.

Further studies on the changes in the enzymes absorption spectrum have been undertaken. Both succinylhomoserine and succinylserine cause transient quenching of the enzyme's 420 mμ absorption band and the appearance of a new band at 480 mμ, but only succinylserine gives the new band at 465 mμ. Alanine and α-aminobutyrate also quench the 420 mμ band but give rise to no new bands. Of special interest was the finding that there are no spectral changes when both cysteine and succinylhomoserine are present. After all the cysteine is consumed in the synthesis of cystathionine (reaction 2), the changes characteristic of γ-elimination occur if succinylhomoserine is present in excess. Similar studies of changes in the enzymes excitation and emission spectra have been initiated.

Attempts to synthesize O-succinyl-γ-hydroxy-valine and O-succinyl-threonine for use as substrate analogs in spectral and kinetic experiments have been unsuccessfull.

Proposed Course of Action:

1. Since the enzyme is known to catalyze γ-replacement, γ-elimination and β-elimination, we are planning to determine whether it also catalyzes β-replacement with succinyl serine as a substrate.

2. We will try to prepare apoenzyme in order to find out if the pyridoxal phosphate is necessary for the β-hydrogen exchange, and also to study the effect of pyridoxal phosphate on the optical rotatory dispersion spectrum of the enzyme.

3. An attempt will be made to correlate the changes in enzyme spectra during various reactions with the results obtained in the experiments with tritium and deuterium water.

4. Kinetic and isotope experiments will be performed in order to better understand the mechanism of γ-replacement and its relation to the now partially elucidated mechanism of γ-elimination.

Publications:

1. Guggenheim and M. Flavin, Proton retention in the γ-elimination reaction catalyzed by cystathionine γ-synthetase. Biochim. et Biophys. Acta, 151(1968) 664.

Serial No. NHI- 9
Laboratory of Biochemistry
Section on Enzymes

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The study of One-Carbon Metabolism

Previous Serial Number: NHI-219

Principal Investigator: L.B. Hersh

Other Investigators: E.R.Stadtman
L.Tsai

Cooperating Units: None

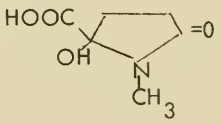
Man Years:

Total:	1.5
Professional:	1.2
Other:	0.3

Project Description:

Objective: To study the reaction between α-ketoglutarate and methylamine catalyzed by cell free extracts of Pseudomonas M.A.

Major Findings: Previous studies in this laboratory have shown that extracts of Pseudomonas M.A. catalyze a reaction between methylamine and α-keto glutaric acid in which the product was tentatively identified as the N-methyl amide of 2-hydroxy-glutaric acid. This product has now been shown to be the N-methyl-5-hydroxy-pyroglutamic acid.



Final proof of this structure was obtained from:

1. The elemental analysis of this compound.
2. Mass spectroscopy
3. The preparation of the proposed compound by reaction of N-methyl-glutamine with L-amino acid oxidase.

Preliminary results suggest that the enzyme catalyzing the formation of the above compound is not identical to the enzyme which catalyzes the formation of N-methyl-glutamic acid from methylamine and glutamic acid.

Using crude extracts of Pseudomonas M.A. we have observed that the methyl group of methylamine is incorporated into alanine and, to a smaller extent, into other amino acids. Work is now in progress to determine whether or not these compounds are derived from N-methyl-5 hydroxy-pyroglutamate.

Proposed course of project

1. To confirm that the reactions of methylamine with glutamic acid and with α-ketoglutaric acid are catalyzed by two different enzymes.
2. To determine the precursor-product relationship between N-methyl 5-hydroxy-pyroglutamic acid and alanine.
3. To investigate any relationshipbetween N-methyl glutamic acid synthesis and N-methyl 5-hydroxypyroglutamic acid synthesis.

Significance to Biomedical Research:

One carbon derivatives are key intermediates in numerous metabolic pathways. The research is a part of a general study aimed at elucidation of biochemical mechanisms involved in the formation and utilization of one-carbon derivatives.

Publications: None

Serial No. NHI- 10
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Carrier-Mediated Transport by Isolated Bacterial Membrane Preparations

Previous Serial Number: 141

Principal Investigator: H.R.Kaback

Other Investigator: None

Cooperating Units: Saul Roseman, Dept. Johns Hopkins University
Roy Vagelos, David Silbert, Frank Ruch, Dept. of Biological Chem.
Washington University, St. Louis

Man Years (computed for the 12 month period)

Total:	1.5
Professional:	1.2
Other:	0.3

Project Description:

Objectives: The mechanisms of carrier-mediated transport by isolated bacterial membranes.

The study is facilitated by the use of mutants deficient in uptake mechanisms for appropriate sugars and amino acids. It is hoped that studies of this model system will ultimately lead to an assay system by which "permeases" may be isolated and their mechanism(s) of action investigated on a biochemical level.

Major Findings:

In 1964, Kundig, Ghosh, and Roseman reported the isolation of a bacterial phosphotransferase system which catalyzes the transfer of phosphate from P-enolpyruvate to various carbohydrates according to the following reactions:

I. P-enolpyruvate + HPr $\xrightarrow{\text{Enz. I, Mg}}$ Pyruvate + HPr-P

II. P-HPr + Sugar $\xrightarrow{\text{Enz. II, Mg}}$ Sugar-P + HPr

HPr, a heat stable, low molecular weight protein, and Enzyme I are predominantly soluble proteins, whereas Enzyme II is membrane bound. Enzyme II is responsible for specificity with respect to the various sugars studied. Subsequently, biochemical and genetic evidence has accrued indicating that this transferase system is involved in bacterial carbohydrate transport in Gram negative as well as Gram positive organisms.

Previous work from this laboratory reported the isolation of a membrane preparation from E. coli which, in the absence of soluble proteins catalyzes the concentrative uptake of proline as well as the facilitated diffusion of glycine and its ultimate conversion to phosphatidylethanolamine. Subsequent studies with sonicated membrane preparations demonstrated that the proline uptake system is tightly bound to the membrane.

The present experiments were conducted with isolated membrane preparations of E. coli using techniques allowing the simultaneous measurement of total radioactive sugar uptake, phosphorylation and the appearance of free sugar in the intramembranal pool. Experiments with membranes prepared from B. subtilis and Salmonella typhimurium have given qualitatively similar results.

Isolated bacterial membrane preparations specifically require P-enolpyruvate for the uptake of certain sugars which accumulate almost completely as phosphorylated derivatives. Furthermore, membranes prepared from E. coli GN-2, a mutant lacking Enzyme I of the P-transferase system, are unable to take up significant quantities of α-methylglucoside.

There is a stoichiometric relation between ^{32}P loss from P-enolpyruvate and appearance of ^{32}P in α-methylglucoside-P.

^{3}H-glucose added to the incubation medium is phosphorylated more rapidly than free ^{14}C-glucose in the intramembranal pool, suggesting that the P-transferase system is a mechanism by which sugars penetrate the membrane. Further evidence for this mechanism is provided by the following observations:

1. The uptake and phosphorylation of α-methylglucoside exhibit saturation kinetics with an apparent K_m of about 4×10^{-6}M, whereas the appearance of free α-methylglucoside in the intramembranal pool shows no saturation (the initial rate of uptake of free α-methylglucoside is independent of the presence of P-enolpyruvate);

2. Under steady state conditions, intramembranal free α-methylglucoside and the external pool do not equilibrate regardless of the presence of P-enolpyruvate; and
3. External α-methylglucoside does not exchange with α-methylglucoside previously taken up by the membranes.

Membranes prepared from glucose-grown cells take up and phosphorylate α- and β-methylglucoside, glucose, 2-deoxyglucose, fructose, galactose, and 3-O-methylglucose in the presence of P-enolpyruvate. Ribose, arabinose, mannitol and sorbitol are not taken up or phosphorylated.

Recently, in collaboration with Dr. Saul Roseman's group at Johns Hopkins University the effects of HPr and Enzyme I on this system have been studies. In the absence of HPr and Enzyme I, the initial rate of α-methylglucoside uptake is linearly related to P-enolpyruvate concentration over a 100,000-fold concentration range. In the presence of HPr and Enzyme I, however, the transport of α-methyl-glucoside is markedly stimulated at low concentrations of P-enolpyruvate. A sigmoid relationship is obtained in which there is no stimulation from 0 to 10^{-4} M P-enol-pyruvate. Above 10^{-4} M, there is a very marked stimulation of uptake to approximately 10^{-3} M P-enolpyruvate. Concentrations higher than 10^{-3} M result in no further stimulation of uptake. Furthermore, it can be seen that the phosphorylation of α-methylglucoside closely mirrors the uptake process. Finally, NaF completely inhibits the stimulatory effect of HPr and Enzyme I on the transport process. Despite this inhibition by NaF on the stimulatory effect of HPr and Enzyme I on transport, phosphorylation still occurs. Under these conditions, sugar-P appears externally rather than within the membrane vesicles. These experiments are consistent with the interpretation that Enzyme II is capable of releasing sugar-P either into the membrane vesicles or into the external medium depending on experimental conditions. This implies that the enzyme has at least some degree of symmetry within the membrane maitrix and that it is capable of undergoing conformational changes.

Studies of the effect of temperature on α-methylglucoside uptake and phosphory-lation by membranes prepared from a variety of strains of E. coli, Salmonella typhimurium, and B. subtilis have demonstrated that the membranes begin to leak sugar phosphates when the temperature is raised above a certain point. Furthermore the point at which they begin to leak differs with membranes prepared from different strains of E. coli and in membranes derived from cultures of B. subtilis grown on either minimal or enriched media. The temperature optimum for phosphorylation (regardless of transport) is at about 46° and is about the same for each of these membrane preparations. Finally, the leokage of sugar-P is due to a rapidly reversible increase in membrane permeability at the higher temperatures. Experiments recently carried out with Dr's Roy Vagelos, David Silbert, and Mr. Frank Ruch at Washington University, St. Louis indicate that the unsaturated fatty acids present in the phosphatides of the membranes may play an important role in these leakage phenomena. Using membranes prepared from their mutant which requires an unsaturated fatty acid for growth,

membranes were prepared from cells grown on oleate (vaccinate is the normal predominant unsaturated fatty acid in E. coli). These membranes were demonstrated to leak at 2-3 times the rate of wild type membranes and furthermore, when examined under the electron microscope, were found to contain a large number of open vesicles.

Proposed Course of Project:

Attempts will be made to correct transport defects in mutants of E. coli and Salmonella typhimurium which have been shown to lack HPr or Enzyme I. Further studies on the effects of HPr and Enzyme I on transport and phosphorylation from the point of view of conformational changes will be carried out using physical techniques (e.g., optical rotatory dispersion, circular dichroism, fluorescent dye studies) which may provide some insight into the nature of these changes. The temperature-induced leakage phenomena will be investigated with respect to the role of fatty acids and proteins in the maintainance of the permeability barrier. Hopefully, methods will be developed by which the membrane-bound component of the P-transferase system (i.e., Enzyme II) can be solublized and studied in free solution.

Publications:

1. Kaback, H.R., and Kostellow, A.B., Glycine Uptake in E. coli I. Glycine Uptake by Whole Cells of E. coli W^+ and a D-Serine-resistent Mutant, J. Biol. Chem. 243, 1384-1390(1968).

2. Kaback, H.R. and Stadtman, E.R., Glycine Uptake in E. coli II. Glycine Uptake, Exchange, and Metabolism by an Isolated Membrane Preparation, J. Biol. Chem. 243, 1390-1400, (1968).

3. Kaback, H.R., The Role of the P-enolpyruvate-P-transferase System in the Transport of Sugars by Isolated Membrane Preparations of E. coli, J.Biol. Chem. (1968) in press.

Serial No. NHI- 11
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Reactions and Enzymes Mediating the Biological Transfer of Sulfur Between Cysteine and Homocysteine

Previous Serial Number: NHI 221

Principle Investigator: Douglas Kerr
Other Investigators: Martin Flavin

Cooperating Units: none

Man Years:

Total:	1.3
Professional:	1.0
Other:	0.3

Project Description:

Objectives: The objective of this study has been to clarify the metabolic pathway by which sulfur is incorporated into methionine in Neurospora. Genetic evidence in Neurospora as well as analogy to bacteria suggested the sequence: sulfide→cysteine→cystathionine→homocysteine→methionine in which the key process is trans-sulfuration from cysteine to a four carbon amino acid. Previous work in this laboratory established that: 1) O-acetylhomoserine is the four carbon precursor, and 2) there is an enzyme in Neurospora (missing in a methionine auxotroph) which cleaves cystathionine to homocysteine. Initial attempts to show the missing step, enzymatic synthesis of cystathionine from cysteine and O-acetylhomoserine, had not been successful. In fact, the finding of an in vitro reaction, sulfide + O-acetylhomoserine → homocysteine, prompted speculation that cystathionine is not a necessary intermediate. Since this would not account for the in vivo requirement for the cystathionine cleavage enzyme, we continued to look more carefully for an enzyme which would synthesize cystathionine from cysteine and O-acetylhomoserine.

Major Findings: It was confirmed that cystathionine is an intermediate in methionine biosynthesis in Neurospora. Trial of a variety of extraction procedures led to the discovery of an unstable enzyme in Neurospora which synthesizes cystathionine from cysteine and O-acetylhomoserine. The rate appears sufficient to account for methionine

biosynthesis in vivo. The enzyme is quite specific for O-acetylhomoserine, showing little or no activity with homoserine or O-succinylhomoserine (the corresponding intermediate in bacteria).

Mutants of two unlinked genes were found to lack cystathionine synthase. These mutants, me-3 and me-7, were previously shown to be able to grow on cystathionine but not on cysteine plus 0-acetylhomoserine. This is particularly interesting since the corresponding enzyme in Salmonella is controlled by one gene and appears to be composed of identica' subunits. When extracts from the two types of Neurospora mutants were mixed activity was restored. This indicates that there are two gene products which can be recombined in vitro. The nature of these two components is not known except that they are both macro-molecular. Enzyme activity is lost upon dilution suggesting that the two components may function as a complex.

A protein has been purified 500 fold from Neurospora which it was thought might be one of these components. This is a pyridoxal phosphate enzyme, 0-acetylhomoserine sulfhydrylase, which forms homocysteine from 0-acetylhomoserine and sulfide. It is also very specific for 0-acetylhomoserine, but it differs from cystathionine synthetase in that it reacts with sulfide and not cysteine. Cystathionine synthase from Samonella reacts with either cysteine or sulfide. The sulfhydrylase is present in both me-3 and me-7 mutants and addition of purified wild type sulfhydrylase does not restore cystathionine synthase activity to either type of extract. Therefore, the function of the sulfhydrylase remains unknown, although the present evidence does not completely rule out a relationship to cystathionine synthase.

When Neurospora is grown in a media containing excess methionine an inhibitor of cystathionine synthase accumulates. The inhibitor has not been identified but is not methionine itself and might be S-adenosylhomocysteine.

Some progress has been made in stabilizing the enzyme which will facilitate further studies.

Significance to Biomedical Research: The general biomedical implications of this project are: 1) methionine is essential for higher animals and the natural cycle of its synthesis in lower organisms is not well understood; and 2) the finding that one enzyme is governed by two genes and also subject to feedback inhibition offers another opportunity to advance our knowledge of biological control mechanisms.

The role of sulfur amino acid metabolism in vascular disease, while little studied to date, is suggested by the early thromboembolic episodes which occur in the inherited disorder, homocystinuria, a defect in cystathionine synthesis.

Proposed Course of Action:

The most interesting aspect of the problem is the unknown function of the two components of what appears to be a single enzyme. It is hoped to characterize at least one of these components by use of partial reactions or demonstrating a requirement for pyridoxal phosphate. It should be possible to conclude more definitely whether O-acetylhomoserine sulfhydrylase is physically or functionally associated with cystathionine synthase. The function of the second component may be characterized only by its effect on the function of the first component. An attempt will also be made to identify the inhibitor and possibly study its metabolic relation to methionine, particularly in certain regulatory mutants.

Publications:

Kerr, D.S., and Flavin, M., Biochem. Biophys. Res. Comm. 31 124 (1968).

54

Serial No. NHI-12
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the conversion of CO_2 to acetate by Clostridium thermoaceticum.

Previous Serial Number: 140

Principal Investigator: J. M. Poston

Other Investigators: E.R. Stadtman

Cooperating Units: None

Man Years

Total:	1.4
Professional:	1.1
Other:	0.3

Project Description:

Objectives; The thermophilic obligate anaerobe, Clostridium thermoaceticum, ferments glucose according to the scheme in which two moles of acetate are derived directly from glucose and a third mole is derived from the reduction of the CO_2.

$$C_6H_{12}O_6 + 2\,H_2O \rightarrow 2\,CH_3COOH + 2\,CO_2 + 8\,H$$

$$8\,H + 2\,CO_2 \rightarrow CH_3COOH + 2\,H_2O$$

Sum: $$C_6H_{12}O_6 \rightarrow 3\,CH_3COOH$$

Previous studies in this laboratory have shown that cell-free extracts of this organism can reduce $^{14}CO_2$ to acetate labeled in both carbons. It has also been established that Co-$^{14}CH_3$-cobalamin can be converted to $^{14}CH_3COOH$ by cell-free extracts and that these extracts can reduce CO_2 to yield Co-methyl cobalamin in the presence of reduced cobalamin (B_{12s}). The conversion of Co-methylcobalamin to acetate is sensitive to sulfhydryl poisons and is dependent upon the presence of coenzyme A and pyruvate and three protein fractions separable by DEAE-cellulose chromatography, fractions A and B and ferredoxin.

55

The objectives of the present investigation have been to examine the reductive processes involved in the conversion of Co-methyl cobalamin to acetate and to purify the enzymic activities associated with these processes.

Major Findings:

As reported previously, pyruvate and CoA are required for the conversion of Co-methyl cobalamin to acetate. Examination of the protein fractions A, B, and Ferredoxin that are required to carry out this conversion showed that fraction A possesses strong pyruvate dehydrogenase activity and is able to reduce low potential dyes such as methyl viologen when supplemented with pyruvate and CoA. It was found that Evan's blue is an effective electron acceptor and, since the reduction products do not yield colored compounds upon exposure to air, the use of Evan's blue permits a sensitive, convenient, and simple assay of enzymic activity.

It was found that TPNH could replace the requirements for pyruvate and CoA, but only if oxidized DPN was present. It was found that the ability to reduce Evan's blue was lost upon dialysis against KBr. Addition of boiled cell extract of C. thermoaceticum restored the ability and it was found that FAD and Mn^{++} was as effective as boiled cell extract. When this protein fraction was supplemented with a TPNH generating system and saturating levels of FAD and Mn^{++}, the requirement for oxidized DPN disappeared. (This DPN requirement was not exhibited for the reduction of methyl viologen.)

The protein fraction purified using the Evan's blue assay does not convert Co-methyl cobalamin to acetate by itself nor does it completely replace either fraction A or fraction B. However, when systems capable of carrying out the conversion at low rates are supplemented by the addition of the Evan's blue fraction, considerable stimulation is observed.

Proposed Course of Action:

Efforts will be made to purify the activity responsible for the reduction of the Evan's blue and to characterize the reaction associated with this activity in the conversion of Co-methyl cobalamin to acetate.

Significance to Biomedical Research: In many pathways of metabolism one-carbon derivatives are important intermediates. The identity of all of the activated forms of these one-carbon derivatives is still unknown. Since a major portion of the metabolism of C. thermoaceticum is concerned with the utilization of one-carbon derivatives, this organism offers a convenient medium for studying the biochemical mechanisms involved. Furthermore, the involvement of vitamin B_{12} in the one-carbon metabolism of C. thermoaceticum has yielded another tool for studying the mechanisms of action of this vitamin.

Results of these studies may be significant to an understanding of the biochemical bases of various physiological disorders associated with vitamin B_{12} deficiency diseases.

Publications : None

Serial No. NHI-13
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: 1) Electron transport systems in anaerobic bacteria
2) Use of mutants in the investigation of the amino acid reductase systems in Clostridium sticklandii

Previous Serial Number: 138

Principal Investigator: Arnold C. Schwartz

Other Investigator: Thressa C. Stadtman

Cooperating Units: None

Man Years:

Total:	1.4
Professional:	1.1
Other	0.3

Project Description:

Objectives: 1. Nature of the electron transfer processes involved in the reduction of proline by Clostridium sticklandi and Clostridium ientoputrescens, and the question of coupling to an ATP-forming mechanism.

2) Application of mutant induction techniques on anaerobic bacteria in the newly developed anaerobic laboratory.

3) Isolation and characterization of mutants affecting the glycine, lysine, and proline reductases in Clostridium sticklandi.

Major Findings:

1. Electron transfer The ability to use NADH or NADPH as electron donors for proline reduction resides in the same partially purified fraction (0.3-0.5 saturated ammonium sulfate (AS) precipitate of the DEAE cellulose effluent of the crude extract) as the proline reductase activity itself. Differentiation between these activities is possible by assaying the proline reductase alone with dithiothreitol (DMT) as a

H-donor (see earlier reports). The dehydrogenases active on NADH and NADPH can be linked to triphenyltetrazolium chloride (TTC) as hydrogen acceptor. TTC inhibits proline reduction to a higher degree than does oxygen. The complete separation of the proline reductase from the NAD dehydrogenase activities proved to be difficult. The ratio of NADH-linked to DMT-linked activity was lowered from 0.66 to 0.12 by passing the fraction over a G-200 gel column, and to less than 0.033 using an agarose A 1.5 column. Both treatments resulted in considerable purification of the proline reductase, the agarose releasing the enzyme in higher yield and in a single peak, but in a polymerized form as indicated by its position close to the exclusion volume. The molecular weight of the enzyme in the ammonium sulfate fraction was determined by sucrose density gradient centrifugation and found to be ca. 400,000.

Synthesis of ATP coupled to electron transport was investigated with dialyzed crude extracts both of C. sticklandii and C. lentoputrescens using ethanol, or isopropanol, each with alcohol dehydrogenase as an NADH generating system. A certain amount of P^{32} esterification (ratio to proline reduced 0.4) was observed with C. lentoputrescens but C. sticklandii showed only traces, when ethanol was used as a donor. However, P^{32} esterification was not observed, when the NADH generating system contained isopropanol. Using ethanol, the ATP formed corresponded with an equal amount of acetate formed. Thus, P^{32} esterification in these preparations seem to be due to a side reaction yielding acetylphosphate.

2) In order to obtain a new line of approach in the investigation of the amino acid degradation performed by C. sticklandi, mutation techniques were applied to these problems. This was made possible by the availability of the anaerobic laboratory. As anticipated, the conditions of inducing mutants with NG can be applied unmodified on clostridia, as long as it is certain that the oxygen concentration is really low during the critical operations (about 10 ppm). Besides it is also necessary to keep the cells in media containing reducing agents whenever possible. The peniciline selection, which requires growing the cells in diluted culture did not work successfully so far.

3) The mutants desired were selected by applying the principle of small colonies. According to this, mutations in the pathways of energy-yielding reactions, which all the amino acid reductases are considered to be, lower the total amount of ATP formed. This in turn decreases the growth of single colonies to a similar degree. Of the survivors from NG-treated suspensions 40-50% were small colonies. Several of the isolated strains showed decreases from 70 to 90% in the activities of one single enzyme or combinations of lysine, glycine or proline reductase.

Proposed Course of Project:

1. The work on the electron transfer will be concentrated on the methyl viologen-linked reactions.

2. More mutants will be isolated and the exact nature of their lesions established, in order to provide a broad selection for biochemical work on the amino acid reductases.

Publications None

Serial No. NHI-14
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Regulation of Glutamine Synthetase in Escherichia coli.

Previous Serial Number: 132

Principal Investigator: Bennett M. Shapiro

Other Investigators: Henry Kingdon
E.R. Stadtman

Cooperating Units: none

Man Years:

Total:	1.5
Professional:	1.2
Other:	0.3

Project Description:

Objectives: An investigation of the means by which the glutamine synthetase of E. coli is regulated has led to the discovery of two additional enzymes which modify glutamine synthetase: (1) adenylylating enzyme that attaches the adenylyl moiety of ATP to glutamine synthetase; and (2) a deadenylylating enzyme that removes the adenylyl moiety. Hence, current studies are directed toward understanding the structural and catalytic features of all three enzymes, as well as the physiologic conditions of the organism which control the relative abundance and activity of these enzymes.

Major Findings: Results of in vivo studies indicate that the E. coli glutamine synthetase undergoes adenylylation and deadenylylation reactions in response to the state of nitrogen nutrition. These conclusions are supported by the demonstration in crude extracts of E. coli of (1) a specific adenylyltransferase, that catalyses the covalent binding of the AMP moiety of ATP to glutamine synthetase, and, (2) a deadenylylating enzyme, that catalyses the removal of AMP from glutamine synthetase. As a result of the activity of these two enzymes, glutamine synthetase as isolated from E. coli has varying amounts of AMP covalently bound to it; adenylylation may involve the attachment of up to 1 AMP per subunit, or 12 moles of AMP per mole of native, dodecameric enzyme. The attachment of AMP converts glutamine synthetase from a form requiring Mg^{2+} for activity to a form which requires

Mn^{2+}. Also, with adenylylation, the enzyme becomes intrinsically less active and exquisitely sensitive to feedback inhibition by many of the products of glutamine metabolism. In addition, there is a change in the pH optimum for the γ-glutamyl transfer reaction catalysed by the enzyme, from 6.9 to 8.0. The quarternary structures of adenylylated and unadenylylated enzymes are apparently identical, as judged by electron microscopy, sedimentation velocity, sedimentation equilibrium, and viscosity experiments.

To determine which amino acid at the modifier site of glutamine synthetase is involved in the covalent attachment of adenylyl groups, ^{14}C-adenylyl enzyme was prepared by incubating unadenylylated glutamine synthetase with ^{14}C-ATP in the presence of glutamine and a partially purified preparation of the adenylyltransferase. Studies with the ^{14}C-adenylyl-labeled enzyme thus produced showed that the adenylyl-enzyme phosphodiester linkage is extremely stable, resisting cleavage in 1 M NH_4OH for 5 hours at 100° or hydrolysis in 2 N HCl for 18 hours at 37° (the latter treatment results in cleavage of the adenine-ribose bond). After proteolytic digestion of the labeled adenylylated enzyme with a combination of pepsin followed by pronase, an AMP containing decapeptide was isolated with the following composition: ($asp_2glu_2pro_3gly_1leu_1tyr_1$). The only residue in this peptide having a functional group that could react with an adenylyl group to produce a phosphodiester of the required stability is tyrosine. Proof that the adenylyl group was bound to a tyrosine hydroxyl group was obtained by comparing the spectra of adenylylated and unadenylylated peptides. Tyrosine has a characteristic 293 mμ spectral peak at alkaline pH that is absent when the phenolic hydroxyl group is bound in ester linkage. When we examined the ultraviolet absorption spectrum of the decapeptide which had been deadenylylated with snake venom phosphodiesterase, there was a spectral peak at 293 mμ at pH 13; this peak was absent in the decapeptide which still contained a covalently bound adenylyl group. From the height of this spectral peak we could calculate that the amount of tyrosine hydroxyl group exposed was equivalent to the amount of AMP bound initially to the peptide. Thus, the hydroxyl group of a tyrosyl residue in glutamine synthetase appears to be the novel functional group involved in the adenylyl-enzyme phosphodiester bond.

The enzyme which adenylylates glutamine synthetase, ATP: glutamine synthetase adenylyltransferase, requires Mg^{2+} for activity, is stimulated by glutamine and inhibited by glutamate. More recently, an enzyme has been found which deadenylylates glutamine synthetase. This deadenylylating enzyme has been purified some 4 fold, and has the following characteristics: (1) it is absolutely dependent upon the presence of a divalent cation (Mg^{2+} and Mn^{2+} are effective) (2) it is also absolutely dependent upon α-ketoglutarate for activity; (3) it is inhibited by glutamine; (4) it is markedly stimulated by nucleotides. The nucleotides which are effective as stimulators of the enzyme are UTP, CTP, ATP, ADP, CDP, poly U, and 5 S RNA; their relative effectiveness diminishes in the order listed. A combination of UTP and CTP is more effective than either one alone. Inorganic phosphate increases the stimulation seen with UTP, but has no effect upon the stimulation by CTP. The nucleotide effects are rather specific with no other mononucleotide or polynucleotide fractions other than those listed being stimulatory.

The effects of α-ketoglutarate, glutamate, and glutamine on adenylylation and deadenylylation of glutamine synthetase have led to the following proposal for the regulation of glutamine synthetase activity by the state of nitrogen nutrition of E. coli. Under conditions of nitrogen excess, there would be little need for glutamine synthesis in the face of high levels of the products of glutamine metabolism. Accumulation of glutamine, secondary to the controls on the biosynthetic pathways which utilize glutamine, would lead to inhibition of the deadenylylating enzyme, and activation of the adenylylating enzyme. Thus, glutamine synthetase would be present in its less active, adenylylated form, which is additionally exquisitely sensitive to feedback inhibition by the products of glutamine metabolism. In this fashion, glutamine directs a decrease in its own biosynthesis. Conversely, with nitrogen starvation, there would be a relative accumulation of α-ketoglutarate and glutamate, leading to activation of the deadenylylating enzyme and inhibition of the adenylylating enzyme. Glutamine synthetase then would be present in the active unadenylylated form, which could channel nitrogen presented to the cell into biosynthesis with high efficiency.

Significance to Biomedical Research: The regulation of enzyme activity is a central problem in cellular metabolism. Consequently, an understanding of enzyme regulation should lead to an appreciation of the spatial and temporal ordering of cellular events, both in the controlled metabolic states of growth and development, and also in the uncontrolled metabolic states of neoplastic growth and malignancy. The study of the regulation of glutamine synthetase in E. coli has led to the discovery of a unique form of enzyme regulation, modification of preformed enzyme by a covalent alteration in its structure, in this case, by adenylylation. This discovery was made possible by using E. coli as a source of copious quantities of enzyme, yet there can be little doubt that the principles which emerge from this study will have general applicability throughout the biologic domain.

Proposed Course of Research

The response of the three enzymes involved in regulation of glutamine synthetase activity to variations in the physiologic state of E. coli will be pursued, both by studies of alterations in growth conditions and alterations in the genetic constitution of the organism. Further attempts to modify the structure of glutamine synthetase will be made, by the use of specific chemical agents, in an attempt to understand the mechanism of the complex feedback inhibition of the enzyme. The role of adenylylation in dramatically altering the kinetic characteristics of glutamine synthetase will be assessed by physicochemical techniques, to try to understand the precise structural basis for the catalytic alterations.

Publications

1. Bennett M. Shapiro, Henry S. Kingdon and E.R. Stadtman, Regulation of Glutamine Synthetase Synthetase VII. Adenylylglutamine Synthetase: A New Form of the Enzyme with Altered Regulatory and Kinetic Properties, Proc. Natl. Acad. Sci., 58 p 642-649 (1967).

2. Henry S. Kingdon Bennett M. Shapiro, and E.R. Stadtman, Regulation of Glutamine Synthetase VIII. ATP: Glutamine Synthetase Adenylyltransferase, an enzyme that Catalyzes alterations in the Regulatory Properties of Glutamine Synthetase, Proc. Natl. Acad. Sci. 58, p 1703-1710 (1967).

3. Bennett M. Shapiro and E.R. Stadtman, Regulation of Glutamine Synthetase IX. Reactivity of the Sulfhydryl Groups of the Enzyme from Escherichia Coli, J.Biol. Chem. 242 No.21 p 5069-5079, (1967).

4. Bennett M. Shapiro and E.R. Stadtman, Glutamine Synthetase Deadenylylating Enzyme, Biochem. Biophys. Res. Commun. 30, 32, (1968).

5. E.R.Stadtman, Bennett M. Shapiro, Henry S. Kingdon, C.A. Woolfolk and Jerry S. Hubbard, Cellular Regulation of Glutamine Synthetase Activity in Escherichia Coli, Advances in Enzyme Regulation (ed.) G. Weber, 1968 in press.

6. Bennett M. Shapiro and Ann Ginsburg, Effects of Specific Divalent Cations on Some Physical and Chemical Properties of Glutamine Synthetase from Escherichia coli: Taut and Relaxed Enzyme Forms., J. Biol. Chem. 1968, in press.

7. R.C. Valentine, B.M. Shapiro and E.R. Stadtman, Regulation of Glutamine Synthetase XII: Electron Microscopy of the Enzyme from Escherichia coli, J. Biol. Chem. 1968 in press.

8. Bennett M. Shapiro and E. R. Stadtman, 5' Adenylyl-O-Tyrosine: The Novel Phosphodiester Residue of Adenylylated Glutamine Synthetase from Escherichia coli, J. Biol. Chem. 1968 (in press).

53

Serial No. NHI- 15
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHI
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of the Fatty Acid Kinase Reaction.

Previous Serial Number: 139

Principal Investigator: P.Z. Smyrniotis

Other Investigators: E.R. Stadtman

Cooperating Units: none

Man Years Total:

Total:	1.4
Professional:	1.1
Other	0.3

Project Description

Objectives: Kinases catalyzing the reversible transfer of the terminal phosphoryl group from ATP to the carboxyl groups of short chain fatty acids forming the corresponding acyl-phosphate derivatives are widely distributed in microorganisms, where they have important roles in energy metabolism. The present project is concerned with the isolation of some of these kinases from Clostridium glycolicum. The purified enzymes will be used to study the mechanism of the phosphoryl group transfer reaction and also for the quantitative analysis of short chain fatty acids in biological materials and enzyme reaction mixtures.

Major Findings:

Clostridium glycolicum catalyzes the ATP dependent phosphorylation of acetate, propionate, butyrate, valerate, caproate and octanoate.

A sixty fold purification of the butyrate kinase activity has been obtained by means of standard protein purification procedures, including treatment with streptomycin,

64

precipitation with alkaline ammonium sulfate and selective adsorption elution from calcium phosphate gel. The presence of more than one kinase activity is indicated by the fact that with purification the ratio of acetate kinase to butyrate kinase activity varies from 5:1 to 0.5:1. Of particular interest was the discovery that both acetate and butyrate kinase activities of the partially purified enzyme are markedly stimulated by carbon dioxide.

Proposed Course of Project:

Efforts to obtain the butyrate and acetate kinases in pure forms will be continued. Eventually, studies on the mechanism of action of the enzyme will be made and attempts will be made to determine the role of carbon dioxide in activation of the enzymes.

Publications None

Serial No. NHI- 16
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHI
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: 1) Anaerobic metabolism of certain amino acids and other nitrogen compounds with especial reference to the role of B_{12}-coenzyme and to electron transfer and phosphorylation reactions involved

2) Metabolism of one-carbon compounds and role of corrinoids in methane biosynthesis.

Previous Serial No. 138

Principal Investigator: T.C. Stadtman
Other Investigators: Arnold Schwartz, (Visiting Scientist, see individual report)
Margaret Grant, (Visiting Scientist since Oct. 1, 1967; see individual report)
Joe N. Davis, Technical Assistant

Cooperating Units: Dr. Lin Tsai (Lab. Biochemistry, NHI)
Dr. J. Retey and Dr. D. Arigoni, Technische Hochschule, Zurich.

Man Years:
Total: 2.8
Professional: 1.5
Other: 1.3

Project Description:

Objectives: 1) Identification and determination of mechanism of B_{12}-coenzyme dependent amino group migration reactions of the lysine fermentation. Purification and characterization of the protein catalysts required. This project carried out jointly with Dr. Margaret Grant since her arrival Oct. 1, 1967.

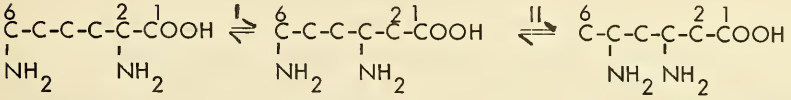
2) Nature of the electron transfer processes involved in the proline and glycine reductase systems of Clostridium sticklandii and Clostridium lentoputrescens. (Major part of the investigations on this project carried out by Dr. Arnold Schwartz).

3) Purification of menadione-dependent p-nitrophenylphosphatase of C. sticklandii and studies on reaction mechanism.

4) Large scale culture of Methanosarcina barkeri on acetate and investigation of nature of conversion of acetate to methane and carbon dioxide.

Major Findings:

1) As described in last year's report, the second enzymic step in the fermentation of α-L-lysine to acetate, butyrate and ammonia involves the B_{12}-coenzyme dependent migration of the terminal amino group of β-lysine forming 3,5-diaminohexanoate (reaction II).



α-L-lysine β-L-lysine 3,5-diaminohexanoate

SCHEME A

$$2\,NH_3 + {}^{6}CH_3{}^{}CH_2{}^{}CH_2COOH + {}^{2}{}^{1}CH_3COOH$$

Reaction I is inhibited by the metal chelating agents, 1,10-orthophenanthroline and α,α-dipyridyl, by alkylating agents such as iodoacetamide, and by the lysine analogue, S-aminoethylcysteine. In the crude system, Reaction II is inhibited by the glycoprotein, intrinsic factor, and by alkylating agents. The sites of action of these two inhibitors are an orange red protein possessing α-(adenyl)-Co-5'-deoxyadenosyl cobamide as chromophore and a second separate protein possessing one or more essential SH groups; both of these proteins are required for catalysis of Reaction II. Fermentation of 3,5-diaminohexanoate to the final fatty acid products is inhibited by low levels of arsenite and requires DPN, an acetyl-CoA generating system, Mg^{++}, Pi and ADP. In crude extracts, the addition of intrinsic factor blocks the fermentation of either α or β-lysine by preventing Reaction II from occurring. However, if 3,5-diaminohexanoate is added as the fermentation substrate, intrinsic factor has no inhibitory effect on its conversion to fatty acids and ammonia. Fermentation of each of the three diamino acids specifically labeled with C^{14} gives rise to the expected labeled fatty acid products as indicated by the numbered carbons in Scheme A. The two dibasic amino acid intermediates are fermented even more rapidly than is α-L-lysine. Synthetic 3,5-diaminohexanoate prepared by Dr. L. Tsai yields one pair of diastereoisomers that contains a compound identical with the enzymic product. By reversal of Reaction II, both the enzymic and synthetic 3,5-diaminohexanoate products can be converted back to β-lysine. The same isomer active in Reaction II is required as substrate for fermentation to fatty acids.

A second B_{12}-coenzyme dependent reaction in the overall lysine fermentation process has also been discovered. In this case the substrate is α-D-lysine which is converted to 2,5-diaminohexanoate.

α-D-lysine

2,5-diaminohexanoate

This reaction is thought to be the first step in Cleavage Process B, wherein acetate is derived from carbon atoms 5 and 6 of the amino acid chain and butyrate from carbon atoms 1 - 4. This overall cleavage has not yet been demonstrated in soluble enzyme systems, presumably due to destruction of one or more labile catalysts. Further studies on this pathway await availability of synthetic 2,5-diaminohexanoate to use as substrate (see Dr. Tsai's report for an account of attempts to synthesize this new amino acid).

The enzyme system catalyzing the migration of the terminal amino group of α-D-lysine, termed α-D-lysine mutase, requires many of the same cofactors needed for the β-lysine mutase system and, like the latter, also consists of two separable proteins. One of these, a sulfhydryl protein fraction, seems to be interchangeable with the sulfhydryl protein component of the β-lysine mutase system. The other, an acidic red cobamide protein fraction, is very similar in properties to the β-lysine mutase cobamide protein. Whereas preparations of the β-lysine mutase cobamide protein plus the sulfhydryl protein component (enzyme 2) catalyze only the β-lysine mutase reaction, all of the cobamide protein fractions active on α-D-lysine are also active on β-lysine. At present it is not known whether two essentially different cobamide proteins are involved or whether a single cobamide protein, modified by reaction with a smaller molecular weight group, is then rendered specific for one of the amino acid substrates. Experiments in progress involving gel filtration techniques and disc gel electrophoresis are aimed at establishing the identity or non-identity of the protein molecules in question.

The α-D-lysine mutase reaction is inhibited by intrinsic factor, activated by B_{12}-coenzyme, and requires ATP, a mercaptan reducing agent, Mg^{++}, and is stimulated by FAD. Unlike the β-lysine mutase reaction, pyruvate is not an activator.

Studies on mechanism of action of B_{12}-coenzyme in the β-lysine mutase reaction continued in cooperation with J. Retey in Zurich, have clearly established that the coenzyme serves a hydrogen carrier for the hydrogen that is removed from carbon 5 and replaced by the amino group. This hydrogen then is transferred back to carbon 6 and appears in the methyl group of the 3,5-diaminohexanoate product. This role as a hydrogen carrier is common to that established for B_{12}-coenzyme in 4 other known reactions.

2) Electron transport coupled to proline reduction: A convenient and satisfactory assay for the additional proteins needed to link proline reductase to reduced methyl viologen as electron donor has been devised. Certain crucial reactants, one of which is the highly autooxidizable reduced dye, are added under anaerobic conditions in the anaerobic laboratory. Partially fractionated extracts utilize reduced methyl viologen even more efficiently than other electron donor systems previously investigated. Stability properties of the system offer promise that the electron transport chain can be reconstituted.

3) Preliminary studies with approximately 50% pure p-nitrophenylphosphatase of C. sticklandii and P^{32}-labeled p-nitrophenylphosphate indicate that P^{32}-labeled enzyme is formed and can be separated from low molecular weight reactant and products by gel filtration. Purification procedures have been further developed for isolation of substrate amounts of enzyme.

4) An assay method for methane biosynthesis by cells and extracts of Methanosarina barkeri has been devised that can be carried out as simple test tube experiments instead of using a more cumbersome manometric apparatus. The oxygen-sensitive enzymes and other reaction mixture components are added, in the anaerobic laboratory, to test tubes which are then closed with serum ports. During incubation of these samples in the ordinary laboratory, aliquots of the gas phase are withdrawn with syringes and assayed for methane. In this system preliminary results indicate that, in the absence of H_2, extracts as well as frozen cells can ferment acetate to $CH_4 + CO_2$. A combination of extract plus a small aliquot of cells gave much more than additive activity. The nature of this activation will be investigated.

Proposed Course of Research

1. Finish the purification and characterization of β-lysine mutase and α-D-lysine mutase component proteins. Study nature of binding of B_{12}-coenzyme to the cobamide proteins and nature of interaction of SH-protein and cobamide proteins. Are there two different cobamide proteins or a single protein that is modified to render it specific for the two amino acid substrates?

To study further metabolism of 2,5-diaminohexanoate and look for missing step in the overall cleavage process. Need chemically prepared 2,5-diaminohexanoate (preparation attempted by Dr. L. Tsai).

To determine stereochemistry of amino group migration reactions of the lysine fermentation as means of further understanding reaction mechanisms.

2. Characterize proteins and cofactors needed to link proline reduction and reduced methyl viologen oxidation. These experiments conducted in anaerobic laboratory.

3. Labeling experiments with P^{32}-labeled p-nitrophenyl phosphate to determine the nature of the interaction between this substrate and the menadione-dependent p-nitrophenyl phosphatase of Clostridium sticklandii. Conditions for maximum formation of P^{32}-labeled protein will be sought and chemical properties of the labeled group of the protein will be investigated.

4. Study of the acetate cleavage reaction to $CH_4 + CO_2$ by extracts of Methanosarcina barkeri. What cofactors are required?

Publications

Stadtman, T.C. and Tsai, L., A cobamide coenzyme-dependent migration of the ϵ-amino group of D-lysine. Biochem. Biophys. Res. Comm. 28:920 (1967).

Bray, R.C. and Stadtman, T.C., Anaerobic degradation of lysine III. N15 studies on the conversion of lysine to 3,5-diaminohexanoate, J. Biol. Chem. 243:381 (1968).

Tsai, L. and Stadtman, T.C., Anaerobic degradation of lysine IV; Cobamide coenzyme dependent migration of an amino group from carbon 6 of β-lysine to carbon 5 forming a new naturally occurring amino-acid, 3,5-diaminohexanoate. Archives Biochem. Biophys. Vol. 123 (1968).

Stadtman, T.C. and Renz , P., Anaerobic degradation of lysine V. Some properties of the cobamide coenzyme dependent β-lysine mutase of Clostridium sticklandii. Archives Biochem. Biophys. Vol. 123 (1968).

Stadtman, T.C. Energy Mechanisms in Anaerobic Bacteria, Bulletin of University of Montreal, School of Microbiology and Hygiene (in press). 1968

Stadtman, T.C., Methane Fermentation, Ann. Rev. Microbiology 21:121 (1967).

Serial No. NHI-17
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Regulation of Glutamine Synthetase Activity

Previous Serial Number: None

Principal Investigator: E.R.Stadtman

Other Investigators: B.M. Shapiro
A. Ginsburg

Cooperating Units: None

Man Years:

Total:	0.8
Professional:	0.5
Other:	0.3

Project Description:

Objective: To determine the effect of adenylylation on the subunit interactions of E. coli glutamine synthetase

Major Findings:

Previous studies have shown that glutamine synthetase from E. coli is an aggregate molecule, composed of 12 apparently identical subunits. Under conditions of nitrogen abundance a specific adenylyltransferase can catalyzes the covalent attachment of the adenylyl moiety of ATP to each one of the subunits. This adenylylation results in a marked change in the pH optimum, the divalent ion specificity and in the catalytic capacity of the enzyme and in its susceptibility to feedback inhibition by multiple end products of glutamine metabolism. Since the enzyme contains 12 subunits arranged in two superimposed hexagonal layers, it is evident that the adenylylation reaction may lead to the formation of molecules having 0 to 12 adenylyl groups per mole. In effect then partial adenylylation of the enzyme can lead to at least 12 different isoenzymes differing from each other in the number of adenylyl groups baund per mole, and a large number of additional isomeric forms can exist depending upon the relative orientation of the adenylyl groups in molecules containing more than one such group per mole.

In view of these considerations, it was of interest to determine if the catalytic characteristics of partially adenylylated molecules of enzyme are those expected from their contents of fully adenylylated and unadenylylated subunits. This knowledge could have a significant bearing on current theories that attempt to explain the regulatory behavior of enzymes on the basis of subunit interaction.

To investigate this problem, fully adenylylated and unadenylylated enzyme preparations were mixed together in various propertions to give solutions of enzyme in which the average concentrations of adenylylated subunits varied from 10 to 100%. The catalytic properties of these mixtures were then compared with partially adenylylated enzyme preparation obtained by the random removal of adenylyl groups from a fully adenylyloted enzyme by means of controlled hydrolysis with snake venom phosphodiesterase. Thus far, studies of the γ-glutamyltransferase activity indicates that the pH-activity profiles and the extents of activation by Mg^{2+} or Mn^{2+} for preparations having the same number of adenylyl groups per mole, are the same for mixtures of fully adenylylated and unadenylylated enzymes as for the phosphodiesterase treated preparations. These results indicate that, so far as these criteria are concerned, the catalytic activity of each adenylylated and unadenylylated subunit is expressed independently and is not appreciably influenced by the extent of heterologous interaction. This conclusion is valid for the γ-glutamylyltransferase activity with respect to the response of this activity to pH and divalent ion specificity under the specific assay condition selected for study. The generality of this conclusion with respect to other parameters remains to be demonstrated.

Proposed Course of Action:

These studies will be extended to include investigations of the kinetics of the biosynthetic activity of partially adenylylated enzyme preparations and their responses to feedback inhibitors, pH, and divalent ion specificity.

Publications YES (pg.3)

Publications

1. Jerry S. Hubbard and E.R. Stadtman, Regulation of Glutamine Synthetase II. Patterns of Feedback Inhibition in Microorganisms, J. Bacteriology, 93, 1045-1055 (1967)

2. C.A. Woolfolk and E.R. Stadtman, Regulation of Glutamine Synthetase III. Cumulative Feedback Inhibition of Glutamine Synthetase from Escherichia Coli, Archives of Biochem. Biophys. 118, 3736-755 (1967.

3. C.A. Woolfolk and E.R. Stadtman, Regulation of Glutamine Synthetase IV. Reversible Dissociation and Inactivation of Glutamine Synthetase from Escherichia coli by the Concerted Action of EDTA and Urea, Archives Biochem. and Biophys. 122, 174-189 (1967).

4. Jerry S. Hubbard and E.R. Stadtman, Regulation of Glutamine Synthetase V. Partial Purification and Properties of Glutamine Synthetase from Bacillus licheniformis, J.Bacteriology 94, 4 p 1007-1015 (1967).

5. Jerry S. Hubbard and E.R. Stadtman, Regulation of Glutamine Synthetase VI. Interactions of Inhibitors for Bacillus licheniformic glutamine Synthetase, J. Bacteriology Vol. 94 p1016-1024 (1967).

6. Bennett M. Shapiro, Henry S. Kingdon and E.R. Stadtman, Regulation of Glutamine Synthetase Synthetase VII. Adenylylglutamine Synthetase: A New Form of the Enzyme with Altered Regulatory and Kinetic Properties, PNAS 58 p 642-649 (1967).

7. Henry S. Kingdon Bennett M. Shapiro, and E.R. Stadtman, Regulation of Glutamine Synthetase VIII. ATP: Glutamine Synthetase Adenylyltransferase, an enzyme that Catalyzes alterations in the Regulatory Properties of Glutamine Synthetase, PNAS 58, 4 pp 1703-1710 (1967).

8. Bennett M. Shapiro and E.R. Stadtman, Regulation of Glutamine Synthetase IX. Reactivity of the Sulfhydryl Groups of the Enzyme from Escherichia Coli J. Biol. Chem. Vol. 242 p 5069-5079 (1967).

9. Regulation of Glutamine Synthetase X. Effect ofGrowth Conditions on the Susceptibility of Escherichia coli Glutamine Synthetase to Feedback Inhibition Henry S. Kingdon and E.R. Stadtman, J. Bacteriology, Vol. 94 p 949-957 (1967).

10. C.A. Woolfolk and E.R. Stadtman, Cumulative Feedback Inhibition in the Multiple End Product Regulation of Glutamine Synthetase Activity in Escherichia Coli, Biochem. Biophys. Res. Commun. 17 p 313-319 (1967)

Publications cont'd.

11. Bennett M. Shapiro and E.R. Stadtman, Glutamine Synthetase Deadenylylating Enzyme, Biochem. Biophys. Res. Commun. 30, 32, (1968).

12. E.R. Stadtman, Bennett M. Shapiro, Henry S. Kingdon, C.A. Woolfolk and Jerry S. Hubbard, Cellular Regulation of Glutamine Synthetase Activity in Escherichia Coli, Advances in Enzyme Regulation (ed) G. Weber, 1968 p 257-289, in press

13. Bennett M. Shapiro and E.R. Stadtman, 5'-Adenylyl-O-Tyrosine: The Novel Phosphodiester Residue of Adenylylated Glutamine Synthetase from Escherichia coli J.Biol.Chem. (in press) 1968.

14. Henry S. Kingdon, Jerry S. Hubbard and E.R. Stadtman, Regulation of Glutamine Synthetase, XI. The Nature and Implications of a Lag Phase in the Escherichia Coli Glutamine Synthetase Reaction, Biochemistry (1968) in press.

15. R.C. Valentine, B.M. Shapiro and E.R.Stadtman, Regulation of Glutamine Synthetase XII: Electron Microscopy of the Enzyme from Escherichia coli, Biochemistry, (1968) in press.

Serial No. NHI-18
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Metabolic Regulation and Mechanism of Phosphoribosylpyrophosphate Synthetase

Previous Serial Number: None

Principal Investigator: R.L. Switzer

Other Investigator: none

Cooperating Units: None

Man Years (computed for the 12 month period)

Total:	1.3
Professional:	1.0
Other:	0.3

Project Description:

Objectives: The biosynthesis of 5-phosphoribosyl-1-pyrophosphate (PRPP) is the point at which ribose-5-phosphate is removed from the oxidative pentose cycle and utilized for the biosynthesis of purine and pyrimidine nucleotides, tryptophan, histidine, and pyridine nucleotides. Previous investigations in this laboratory have shown the PRPP synthetase of Salmonella typhimurium to be subject to an additive type of feedback inhibition by the end products of PRPP metabolism. In order to obtain a more thorough understanding of the regulation of this enzyme, further kinetic and mechanistic studies have been performed. In addition, the possibility of regulation of PRPP synthetase by repression of enzyme synthesis was investigated.

Major Findings:

Growth of S. typhimurium in the presence of high levels of end products derived from PRPP or starvation for carbon or phosphate source had only slight effects on the level of PRPP synthetase, as compared to the activity of cells grown on a glucose-salts medium. De-repression of the histidine operon or over-production of histidine (and hence PRPP) by a regulatory mutant had no effect on the specific activity of PRPP synthetase. Thus, either PRPP synthetase is a constitutive enzyme, or the nutritional state of the organism has little effect on levels of the co-repressor.

A procedure was devised for 400-450 fold purification of PRPP synthetase from extracts of S. typhimurium. The preparations appear to be nearly homogeneous on disc gel electrophoresis. The purification makes use of the extremely low solubility of the enzyme (about 0.5 mg/ml in 50 mM potassium phosphate, pH 7.5). A general characterization of the enzyme has been carried out.

PRPP synthetase requires Mg^{++} or Mn^{++} ions for activity. The substrate saturation curve for Mg^{++} -ATP is sigmoid, but is converted to a hyperbolic curve by excess Mg^{++} or Mn^{++} ions. Kinetic studies show that both metal ions act by increasing the affinity of the enzyme for the Mg-ATP complex, and that there are at least two sites with different affinities for Mg-ATP. The activation by Mg^{++} or Mn^{++} at low levels of ATP is potentially several hundred fold. It is not clear whether this activation is of significance as a regulatory mechanism.

PRPP synthetase has been found to catalyze two exchange reactions: (1) AMP:ATP exchange in the absence of ribose derivatives, and (2) ribose-5-phosphate: PRPP exchange in the absence of adenine nucleotides. The AMP:ATP exchange reaction requires enzyme, Mg^{++}, and ATP in addition to AMP; it is strongly inhibited by ribose-5-phosphate. The ribose-5-phosphate: PRPP exchange reaction requires enzyme, Mg^{++}, and PRPP as well as ribose-5-phosphate. AMP at 10^{-4} M and below markedly stimulates the ribose phosphate: PRPP exchange; at higher levels of AMP the reaction is very nearly completely inhibited. These exchange reactions suggest that the PRPP synthetase reaction proceeds via an enzyme-pyrophosphate intermediate:

$$\text{Enzyme} + \text{ATP} \rightleftharpoons \text{Enzyme}\sim\text{PP}_i + \text{AMP}$$

$$\text{Enzyme}\sim\text{PP}_i + \text{R-5-P} \rightleftharpoons \text{Enzyme} + \text{PRPP}$$

The formation of an enzyme-pyrophosphate has been demonstrated directly by incubating the enzyme with ATP-γ-^{32}P and separating the products on Sephadex G-25. Label from ATP-γ-^{32}P was incorporated into the enzyme; control experiments with ATP-ul-^{14}C showed that no more than 2-3% of this labeling could have resulted from binding of intact ATP. When ribose-5-phosphate was included in the incubation mixture, virtually no ^{32}P labeled enzyme was obtained, which suggests that the enzyme-pyrophosphate transfers the pyrophosphate to the acceptor. These studies indicate that it should be possible to isolate and chemically characterize the enzyme-pyrophosphate intermediate.

The effects of inorganic phosphate on PRPP synthetase are complex. Removal of phosphate by dialysis brings about a total, irreversible inactivation of the enzyme, both in the overall reaction and in the exchange reactions. Simply lowering the phosphate concentration by dilution partially inactivates the enzyme. Phosphate is required for the synthesis of PRPP, yet the exchange reactions are inhibited strongly by phosphate with a concentration dependence which is very similar to the dependence of

phosphate stimulation on concentration. Thus, phosphate seems to have a role in maintaining the structural integrity of the protein, as well as a role in the enzyme catalysis.

Significance of work for Biomedical Research: The significance of this work for medical science lies in the information which will be obtained about the detailed mechanisms of enzyme catalysis and control in general and about the regulation of nucleic acid biosynthesis in particular. These processes are undoubtedly important in the cellular growth and differentiation of all organisms, so that an understanding of them in bacteria should be useful to human physiology and thereby to medicine. As an example, it is known that a number of metabolic diseases, such as gout, are related to nucleic acid metabolism; the results of this investigation will be useful to workers studying these diseases.

Proposed Course of Project:

(1) The isolated enzyme-pyrophosphate complex will be characterized chemically.

(2) Kinetic evaluation of the proposed mechanism of action of PRPP synthetase will be undertaken. A careful kinetic analysis of the action of feedback inhibitors will be performed, with the object of determining the mechanism of inhibitor action on the enzyme.

(3) Attempts will be made to characterize PRPP synthetase physically and to evaluate the effects of metal ions, inorganic phosphate, pyrophosphorylation by ATP, and feedback inhibitors on the protein structure.

Publications: None

77

Serial Number NHI-19
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: 1) Metabolism of Heterocyclic Compounds - Chemistry.
2) Application of Physical Methods in the Determination of Structures of Organic Compounds.
3) Synthetic Studies of Organic Compounds of Biological Interest

Previous Serial No: 135

Principal Investigator: L.Tsai

Cooperating Units: E. Charney, LPB, NIAMD (Project 2(A)).
R.J.Highet, SC, LM, NHI (Project 2)B)).

Man Years
Total: 2.5
Professional: 1.2
Other: 1.3

Project Description:

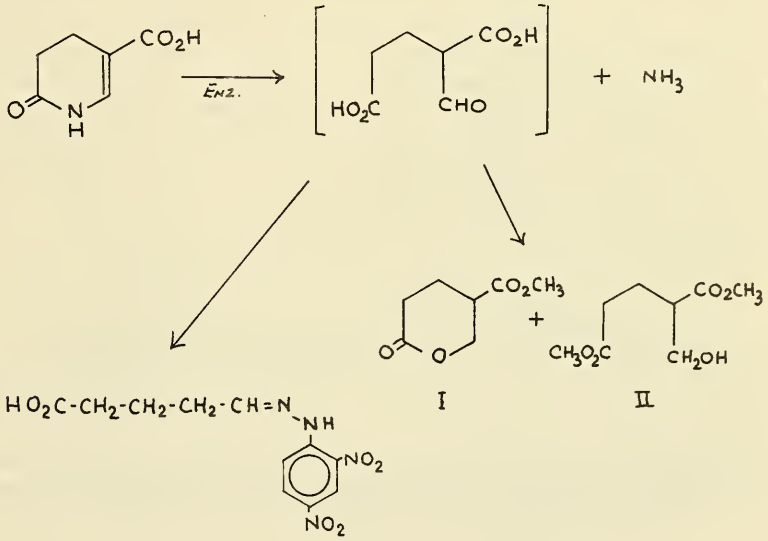
Objectives: To study the chemistry of model compounds and to prepare substrates for the study of metabolism of heterocyclic compounds, and to identify and determine structures of intermediates from metabolic processes.

Major Findings:

The availability of 6-oxo-1,4,5,6-tetrahydronicotinic acid by chemical synthesis permitted studies on the metabolism of this compound. In the presence of an enzyme extract 6-oxo-1,4,5,6-tetrahydronicotinic acid was hydrolyzed to ammonia and α-formylglutaric acid.

The structure of α-formylglutaric acid was deduced from the following observations. When the enzymic reaction was stopped by the addition of 2,4-dinitrophenylhydrazine reagent, a hydrazone derivative was isolated and purified by repeated recrystallizations. Elemental analysis indicated an empirical formula of $C_{11}H_{12}N_4O_6$; the absorption and the nuclear magnetic resonance (NMR) spectra suggested that it was a derivative of an aliphatic aldehyde. The presence of a carboxyl group was demonstrated by the reaction with diazomethane yielding a methyl ester, whose NMR, infrared and mass spectra were consistent for the 2,4-dinitrophenylhydrazone of methyl glutarylsemialdehyde,

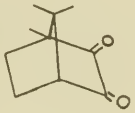
a structure that required the loss of a one-carbon fragment from the starting material. The formation of glutaric acid semialdehyde could be the result of decarboxylation of a-formylglutaric acid under the strong acidic condition employed in the isolation. That this was indeed the case was demonstrated by termination of the enzymic reaction with potassium borohydride followed by conversion of the acidic components to methyl esters by diazomethane. The mixture was analyzed by gas-liquid chromatography (GLC) which showed two major components. Various attempts to isolate these compounds in pure forms were unsuccessful. The mixture was therefore analyzed in a mass spectrometer equipped with GLC. Interestingly these two compounds showed identical mass spectra, whose fragmentation pattern could be accounted for by the lactone-ester structure I. On this basis, the other component could most likely be that of the diester II which upon electron impact lost a molecule of methanol to yield the same ion as that derived from structure I.



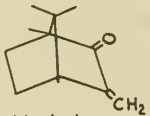
The correctness of the assignment of the structure I to the lactone-ester was shown by catalytic hydrogenation experiments of methyl coumalate which furnished a mixture of two major components by GLC analysis. One of them had the same retention time as well as an identical mass spectrum as the lactone-ester I.

Project Description (2)

(A) Spectroscopic Studies of α-Diketones: Camphorquinone, an optically active α-diketone in which the diketone grouping is held in a rigid conformation, presents itself as a molecule of great theoretical interest for the understanding of the spectroscopic properties of conjugated diketones. Camphorquinone was prepared by the action of



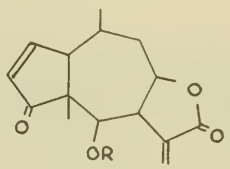
Camphorquinone



3-Methylenecamphor

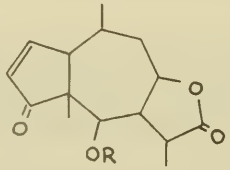
selenium dioxide on camphor. Its visible and ultraviolet spectra, optical rotatory dispersion and circular dichroism curves were determined in solvents of varying degrees polarity. Infrared, Raman as well as emission and excitation spectroscopic data were also obtained. As analgous compounds, camphor and 3-methylenecamphor were studied. 3-Methylenecamphor was prepared by base catalyzed condensation of camphor with ethyl formate, followed by reduction with potassium borohydride and elimination of water by alcoholic potassium hydroxide. Consideration of all the experimental data and the theoretical treatment of the molecular orbitals of the conjugated diketone led to the assignment of the 480 mμ-band of camphorquinone to an electronically allowed $n_1 \rightarrow \pi_3$ transition combined with a vibronic component of the $n_2 \rightarrow \pi_3$ transition, while that of the 280 mμ-band to a $n_1 \rightarrow \pi_4$ transition. This study also indicates that the four atoms involved in the conjugated diketone system are at least slightly skewed out-of-plane.

(B) Mass Spectroscopic Study of Sesquiterpenoid Lactones: Continuation of the mass spectroscopic study of sesquiterpenoid lactones led to the recognition of characteristic peaks at m/e 95, 96, 122, 123 and 124 for compounds having structural features A and B. High-resolution studies revealed that peaks at m/e 123 and 124 were doublets (C_8O and C_7O_2 fragments) for the A-type compounds and essentially singlets (C_8O fragment) for the B-type compounds. A mechanism for the fragmentation that could accommodate these observations was postulated, and the mass spectra of three different deuterium labeled derivatives substantiated the proposed mechanism.



(R = H or Ac)

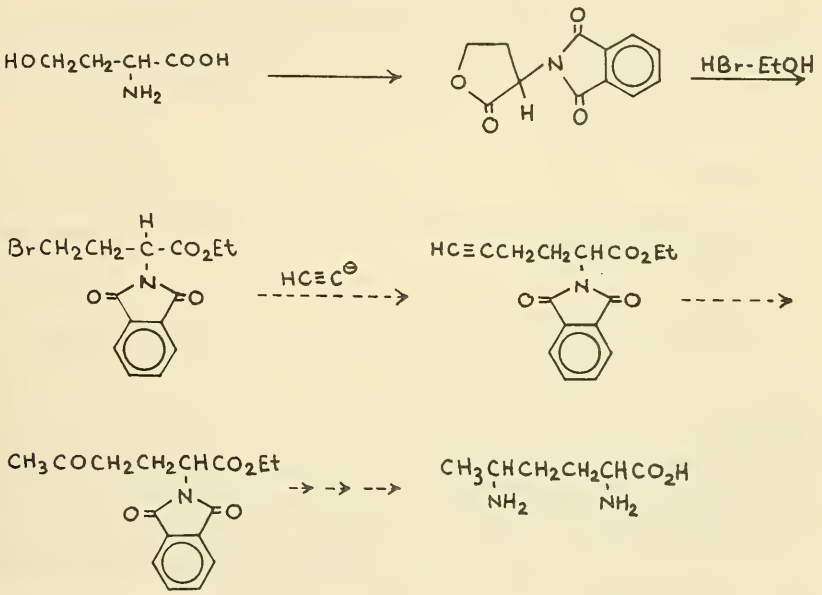
A



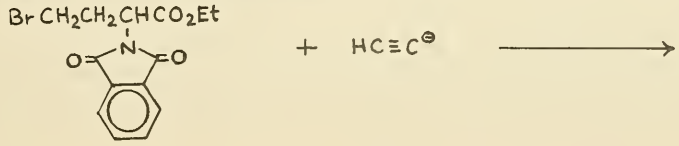
B

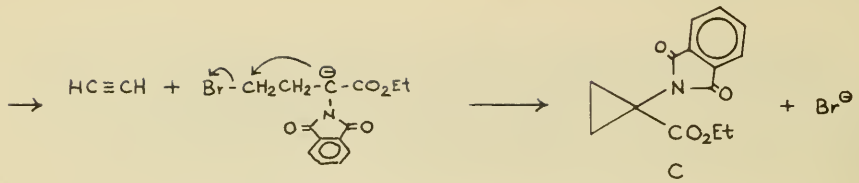
Project Description (3):

2,5-Diaminohexanoic Acid: Studies on the metabolism of lysine by T.C.Stadtman demonstrated that 2,5-diaminohexanoic acid was one the intermediates. In order to provide material for further study, a practical synthesis of this compound is much desired. One approach to this problem is to employ homoserine as the starting material for the sequence of reactions outlined below:



This approach offers the advantage of maintaining the stereochemistry of homoserine throughout the steps, thus the final product would possess an α-amino group of known absolute configuration. Unfortunately, the coupling reaction between the bromo compound and metal acetylide did not proceed in the desired course, instead, a complex mixture was obtained from which a crystalline compound was isolated in pure state. A cyclopropane structure, C, was assigned to this compound on the basis of its NMR, infrared and mass spectra. Its formation could be rationalized as an intramolecular nucleophilic displacement:





C

This rationale was supported by the observation that a much improved yield of the cyclopropane compound was obtained when the bromo compound was treated with sodium ethoxide in anhydrous tetrahydrofuran. Although these experiments failed to achieve the original goal, they have provided a convenient synthesis of a cyclopropane α-aminoacid.

Proposed Course of Action

1. Methods to prepare methyl α-hydroxymethylglutarate and the corresponding lactone ester will be studied.
2. An alternative route to synthesize 2,5-diaminohexanoic acid will be investigated.
3. The spectroscopic study of α-diketones and the mass spectrometric study of organic compounds will be continued.

Publications

1. M.S. Newman, R.S. Darlak, and L. Tsai, Optical Properties of Hexahelicene, J. Am. Chem. Soc., 89, 6191 (1967).

2. Thressa C. Stadtman and L. Tsai, A. Cobamide Coenzyme Dependent Migration of the ε-amino Group of D-Lysine, Biochem. Biophys. Res. Comm., 28, 920 (1967).

3. L.Tsai and Thressa C. Stadtman, Anaerobic Degradation of Lysine IV. The Cobamide Coenzyme Dependent Migration of an Amino Group from Carbon 6 of β-Lysine to Carbon 5 Forming a New Naturally Occurring Amino Acid, 3,5-diaminohexanoate, Arch. Biochem. Biophys., in press.

Serial No. -NHI-20
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

NIH-PHS
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Structure of the Muscle Protein, Myosin.
I. Studies in the Active Site of Myosin.

Previous Serial No.: NHI-232

Principal Investigators: W. Wayne Kielley
Juanita P. Cooke

Other Investigator: Regina A. Grimek

Man Years (computed for the 12 month period):

Total:	2.0
Professional:	1.5
Other:	0.5

Project Description:

Objectives: The continuing objectives of this program are identification of the details of molecular structure of myosin as they relate to the biochemical properties of the protein with particular emphasis on the primary structure in the region of the active site. Previous reports have dealt with studies on subunit structure, physical and chemical homogeneity of the large polypeptide chains, terminal amino acids, and limited amino acid sequences around the fifteen cysteine residues in each polypeptide chain, particularly the two involved in the enzymatic site.

Methods Employed and Major Findings:
Activities during the past year have been directed toward application of the cyanogen bromide cleavage reaction for obtaining larger fragments of the polypeptide chain in order to extend our amino acid sequence information. As indicated in the previous report, complete reaction is obtained in 70% formic acid. The mixture of peptides is largely insoluble in water or neutral buffers but is soluble in 5% formic acid. These circumstances have restricted initial fractionation procedures to gel filtration. Work so far has been focused primarily on gel type, exclusion limits, length of columns, etc. Sephadex G-75 handling molecules up to 50000 in molecular weight gave the broadest distribution and is currently being used for an initial fractionation step giving eight crude fractions with average particle sizes from about 300 to 15000. Further fractionation of those fractions with average molecular weights up to 2000 by ion exchange chromatography and paper electrophoresis demonstrated that some of the smaller peptides occurred in less than molar equivalent amounts. This necessitated a reassessment of the composition of our myosin solutions.

It has been known for some time that all myosin preparations contained low molecular weight protein material more or less tightly associated with the high molecular weight myosin polypeptide chains. In some published work this has accounted for as much as 20% of the protein. While our own preparations have only 7-10% of low molecular weight material, it seemed advisable for a variety of reasons to attempt further purification. However for sequence work on the large polypeptide chains it was observed that these could be obtained pure by fractionation under denaturing conditions, i.e. in the presence of urea or guanidine hydrocholoride.

Introducing a number of modifications into our preparative procedure, controlled precipitions at high and low pH, treatment with ATP, and cellulose phosphate chromatography, we have been able to reduce the amount of this low molecular weight material to 2.5% but have so far not been able to eliminate it entirely.

Returning to the cyanogen bromide reaction, in our initial exploration of suitable solvent conditions for the reaction, we attempted to use 0.1 M HCl for this purpose. Varying reagent concentration, time, and temperature and repeating the reaction we were unable to react more than 70% of the methionine residues. Though this system was discarded as unsuitable, we reinvestigated it when we became aware that light meromyosin (LMM), the highly α-helical tail-portion of the myosin molecule release on brief tryptic digestion, though soluble in dilute HCl is not dissociated in this solvent and that the limitation in the reaction of cyanogen bromide with the methionine residues of myosin might be due to inaccessability of the residues in the LMM portion which possesses about 25% of the total methionine. This has proved to be the case and from cyanogen bromide cleavage in dilute HCl we have isolated material with all the properties of LMM. Since this material contains no homoserine but does contain the anticipated amount of methionine we can conclude that it is possible to obtain this portion of the molecule by this procedure without the broken internal peptide bonds, with resulting loss of small segments, as occurs in preparation of LMM by enzymatic methods. In addition, since homoserine, the product of cyanogen bromide action, always occurs at the carboxyl end of the cleaved polypeptide chain, its absence from LMM prepared in this way confirms our earlier conclusion derived from amino terminal end group analysis that in the match-like structure of myosin the amino terminal ends of the polypeptide chains are in the head and the carboxyl terminal ends are the terminus of the LMM tail. The availability of this type of fragment will greatly facilitate some of the amino acid sequencing objectives particularly those related to the relationship to helix formation, intra and inter molecular association.

Significance to Bio-Medical Research:
This work is aimed at a better understanding of the mechanism of muscular contraction.

Proposed Course of Research:
So far the yields of cyanogen bromide produced light meromyosin have been quite low. In order to use this material for amino acid sequence studies it will be necessary to design procedures to obtain larger quantities.

This material will then be subjected to complete cleavage with cyanogen bromide and the peptide fractionated with gel filtration and ion exchange chromatographic procedures being developed for the CNBr fragments of whole myosin. These parallel procedures will then allow us to sort out the fragments belonging to the heavy meromyosin and "amorphous" regions of the molecule.

Publications:
None.

Serial No.-NHI-21
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The structure of the muscle protein, myosin. II
The binding of nucleosidepolyphosphates by myosin

Previous Serial No.: NHI-233

Principal Investigators: W. Wayne Kielley
T. Yamashita

Man Years: (computed for the 12 month period)

Total:	1.25
Professional:	1.25

Project Description:

Objectives: It has been assumed by many investigators that the two active site sulfhydryl groups in myosin are involved in substrate binding and not in the catalytic process itself. The present project was aimed at an examination of the effect of blocking these SH groups on substrate binding. It was also hoped that some of the current uncertainties concerning the maximum number of binding sites per molecule could be resolved. The previous report presented preliminary results indicating that substrate binding was not influenced significantly by blocking these two sulfhydryl groups. However, maximum binding and binding constants had not been established. This work has been continued. In addition, it was desired to use chemical and enzymatic probes for conformation changes that might be induced by substrates and possible variations in such changes that might be dependent on substrate structure.

Methods Employed and Major Findings:
Binding was determined by equilibrium dialysis in a medium of 0.5 M NaCl and 1 mM EDTA. While myosin is catalytically inactive in this solvent system under the usual conditions of assay, there is a very low rate of hydrolysis of ATP and CTP by native myosin in the 20 hour period required for equilibrium dialysis, sufficient to make the results with these two substrates and native myosin unreliable. Otherwise, this phase of the program has been completed. Briefly stated, sequential blocking of the two active cysteine residues in each subunit polypeptide chain does not alter either the maximum binding nor the dissociation constants of the enzyme substrate complex. The affinity of myosin for the four nucleoside triphosphates studied are in the order: ATP $\gg$ ITP $>$ CTP $\gg$ GTP. ADP is the only diphosphate bound, at least within the concentration range employed, and none of the nucleoside monophosphates is bound. The affinity for ADP is

only about one-twentieth that for ATP. Maximum binding for all the nucleotides approximated one mole per mole of subunit (0.73-0.95 moles/mole subunit). The affect of divalent ions on the binding of ADP was also examined. Ca^{++} had little affect but Mg^{++} increased the affinity for the nucleotide ten fold.

In competitive binding experiments the concentration ratios necessary for 50% inhibition of binding of radioactive ADP or ITP were consistent with the relative magnitudes of the affinity constants.

In the sequential blocking of the two active center sulfhydryl groups, reaction of the second group requires the presence of ATP. As concluded in past reports, it appears that ATP induces a conformational change that lead to this second SH group becoming more reactive. In view of our binding experiments we investigated the capacity of other bound nucleotides to induce these changes. Only ATP, ADP and CTP, nucleotides possessing an amino group in the 6 position of the purine or pyrimidine ring, stimulated the reaction of the second SH with N-ethyl maleimide. ITP and GTP, with an hydroxyl group in the 6 position, had a protective effect. On the other hand, with the 1st sulfhydryl group blocked, the presence of any one of the nucleotides that is bound accelerated the loss of ATPase activity by trypsin or chymotrypsin suggesting that all these nucleotides produced some type of unfolding to expose the peptide bonds susceptible to these two proteolytic enzymes. In the case of chymotrypsin, myosin was particularly sensitive in the presence of ITP.

Significance to Bio-Medical Research:
This work is aimed at a better understanding of the mechanism of muscular contraction.

Proposed Course of Project:
Some of the contemplated projections of this program, the role of divalent metals in binding, and the variation in binding constants with temperature to determine binding energies are dependent on removal of trace interfering enzyme activities or the development of a more rapid method of determine binding than equilibrium dialysis. These latter two problems will be the first order of business.

Publications:
None

87

Serial No.-NHI-22
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Structure and Biochemical Activity of Actin

Previous Serial No.: NHI-234

Principal Investigators: Robert Adelstein
Michael Kuehl
W. Wayne Kielley

Other Investigators: Regina Grimek

Man Years (computed for the 12 month period):

Total:	2.75
Professional:	2.25
Other:	0.50

Project Description:

Objectives: The purpose of this project is to separate and purify the cyanogen bromide fragments of rabbit skeletal actin. The fragments would then be ordered in the sequence they exist in actin. Particular attention would be paid to relating the biological function of the peptide segments, especially the one containing 3-methyl histidine.

Methods Employed and Major Findings:
Classical techniques of peptide separation have been used with particular emphasis on finding a suitable solvent for the large peptides as well as a suitable method of column chromatography. Using a combination of column and paper chromatography we have succeeded in purifying ten of the sixteen peptides including that fragment containing 3-methyl histidine. The other peptides have already been separated on Sephadex chromatography and are ready for final purification.

Significance to Bio-Medical Research:
This work is aimed at a better understanding of the mechanism of muscular contraction.

Proposed Course of Project:
Current separation and purification efforts will be continued and some selective amino acid sequencing of the peptides will be started. It is possible that one or more of the cyanogen bromide peptides will retain some vestige of biological activity such as the ability to combine with myosin or polymerize, which could provide identification of functionally active regions. Other approaches to this problem such as blocking or destruction

of specific amino acid residues will also be explored.

Moreover knowing the composition of the 3-methyl histidine peptide will allow us to pursue some interesting comparative studies. Actin and actin-like proteins are being reported in plethora in the literature recently; from flagella, microtubules, plasmodia and red cells. With Dr. Edward Korn we have succeeded in separating an actin-like protein from platelets and have confirmed the presence of 3-methyl histidine in approximately the same amount as present in actin. It remains to be seen if there will be a similarity in the cyanogen bromide peptides containing 3-methyl histidine and if other actin-like proteins will contain this unique residue.

Publications:
None.

Serial No. -NHI-23
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The biochemistry and cytology of transport

Previous Serial No.: NHI-234

Principal Investigators: Edward D. Korn
M. Blair Bowers
Mary L. Wetzel
Andrew Ulsamer

Other Investigators: Paddy L. Wright (Technician)
Thomas E. Olsewski (Technician)

Man Years (computed for the 12 month period):

Total:	5.67
Professional:	3.67
Other:	2.0

Project Description:

Objectives: To study the biochemical and cytological events of transport across the cell membrane. At this time attention is focused on the process of phagocytosis by the amoeba Acanthamoeba.

Methods Employed:
1) Electron microscopy: Standard techniques of fixation, embedding and sectioning are employed in most studies. Autoradiography is now being attempted. Procedures for the freeze-etching technique are being developed in the laboratory.

2) Phagocytosis: Amoebae are incubated with polystyrene latex beads (usually 1μ in diameter). After uptake has occurred the cells are washed free of excess beads and the latex is extracted with dioxane. The characteristic absorption in the ultraviolet is used to measure quantitatively the rate of phagocytosis.

3) Isolation of phagocytic vesicles: Procedures have been developed for the isolation of phagocytic vesicles from cell homogenates by differential centrifugation in sucrose gradients.

4) Lipid Analyses: Lipids are extracted from cells in the usual way with chloroform-methanol, and fractionated into neutral lipid and phospholipid classes by column chromatography on silicic acid. Individual components are further separated and partially identified by chromatography on thin-layer

silicic acid plates. Phospholipids are analyzed for ester bonds, glycerol, P, and bases by standard methods. Glycerides are analyzed for ester groups and glycerol. Sterols are analyzed by gas-liquid chromatography, mass spectrometry, ultraviolet and infrared spectroscopy and by various techniques of chemical degradation.

5) Membrane turnover: Amoebae are incubated in the presence of a radioactive precursor (usually $^{32}PO_4$) with and without the addition of latex beads. The phospholipids are isolated and their specific activities determined. Measurement of the specific activity of ATP is also made for an internal control of the specific activity of the precursor pool. ATP is isolated by adsorption onto activated charcoal. Radioactivity is measured in a scintillation spectometer.

6) Isolation of actin or actomyosin from amoebae: Attempts have been made to extract actomyosin from amoebae in 0.6 M KCl and precipitate it by dilution of the ionic strength in the presence of Mg. Attempts have also been made to isolate actin directly from acetone-dried powders of amoebae. The actin would be recovered from the solution by specific precipitation by purified skeletal muscle myosin. These procedures have been applied by other investigators to the isolation of actin and actomyosin-like proteins from tissues other than muscle.

7) Isolation of actin from platelets: In collaboration with Dr. Robert Adelstein efforts have been made to isolate actin from human blood platelets. Published procedures of other investigators have been followed for the isolation of a partially purified actomyosin-like protein. This has been disassociated in the presence of high concentrations of ATP, and centrifuged at high speed to give a supernatant solution that is enriched with an actin-like protein. Dialysis against ATP in distilled water leads to a further purification.

Major Findings:
1) The morphology of the amoeba during active growth and throughout the encystment process has been described. The amoeboid form has several interesting features including microtubules and microfibrils, the latter being especially prominent in pseudopods and in the hyaline layer that underlies the plasma membrane. These structures may be of importance in the movements associated with phagocytosis. The morphology of the contractile vacuole, the organelle responsible for water and ion secretion, has also been carefully studied. The fine structure of the cyst wall has been described and the ultrastructural changes that lead to its formation have been delineated. There are major changes in the mitochondria, endoplasmic reticulum and especially the golgi membranes which swell with dense material that is transported through the plasma membrane and deposited as wall. The morphological changes have been correlated with a decrease in cell glycogen, a decrease in phospholipids and an increase in glycerides. Another obvious morphological change is the appearance of autolysosomes which engulf cytoplasmic constituents, glycogen in particular, and degrade them with autolytic enzymes. Food vacuoles are also present in the growing

cells and these seems to be the site of digestion of nutrients. This represents the first reasonably complete study of the ultrastructure of an amoeba.

2) Phagocytic vesicles have been isolated in essentially quantitative yield. Cells are washed free of excess beads and homogenized in 30% sucrose which is then layered under 25, 20, and 10% sucrose. After centrifugation for 45 minutes at 30,000 rpm single beads tightly bound by the phagocytic membrane (plasma membrane) are concentrated between the 10 and 20% sucrose bands. Beads that have entered the digestive vacuoles concentrate between the 20 and 25% sucrose layers. Both fractions are cleanly separated from the major cellular components which remain in the 30% sucrose layer. Free fat floats to the top of the 10% layer. The bead fractions are then collected and diluted with buffer and are readily sedimented by centrifugation. By this procedure and a slight modification of it, phagocytic vesicles are obtained in high purity with little if any contamination by endoplasmic reticulum or mitochondria as monitored by electron microscopy and enzymatic assays. The lighter of the two bead fractions represent beads that have just been ingested and as would be expected these vesicles have a very low concentration of the hydrolytic enzymes, acid phosphatase and glucosidases. The more dense fraction represents beads that have passed from the initial phagocytic vesicle into the digestive vacuole and has a much higher content of hydrolytic enzymes. (These results are still preliminary).

3) The lipid compositions of the whole cell and of the phagocytic vesicles have been defined at least with regard to the major components. The major phospholipids of the cells are phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine in decreasing order. In the membranes the relative amounts of phosphatidyl choline and phosphatidyl ethanolamine are reversed and there is somewhat more phosphatidyl serine than in the whole cells. Both fractions contain 1-4% of a compound that behaves chromatographically very much like cardiolipin but seems to differ from it. Both contain 2-5% lysophosphatidyl choline and about 5% of unknown phospholipids. In addition several iodine positive but P-negative spots are present that may be glycolipids and sulfolipids. No evidence for sphingolipids or inositol lipids has been found.

The neutral lipid of the whole cells is about 65% triglyceride and 25% free sterols with a very small amount of mono- and di-glycerides and sterol ester. The sterol fraction is largely composed of two sterols: ergosterol, 40% and 7-dehydrostigmaterol, 60%. The latter compound has been identified by a number of criteria and it is the first time it has been found in protozoa. The sterol content of the vesicle lipids is about 2-3 times greater than of the whole cells and there is about 0.7μmoles of sterol per μmole of phospholipid. This is a typical value for plasma membranes. The glyceride content varies and may be contaminated by free fat adsorbed during the isolation of the phagocytic vesicles.

4) It has been definitely shown that there is no stimulation of the incorporation of $^{32}PO_4$ into any of the phospholipids of the whole cell or of the membranes of the phagocytic vesicle during active phagocytosis. In fact all of the phospholipids have a lower specific radioactivity than those of the control cells. This decreased specific activity can be entirely accounted for by the lower specific activity of the cellular ATP which is due to the reduced uptake of inorganic phosphate from the medium when phagocytosis is also occurring. Thus with the use of lipid precursors no effect of phagocytosis on membrane turnover or synthesis can be shown.

5) An actin-like protein from human platelets has been prepared from the actomyosin-like protein isolated by published procedures. The best material, obtained in only small yield, may be highly pure judging from the ratio of histidine/3-methylhistidine of 8-9/1 which is very similar to that of pure skeletal muscle actin. These studies have been in collaboration with Dr. Robert Adelstein.

Significance to Bio-Medical Research:
An understanding of the structure and function of the plasma membrane is fundamental to the understanding of many normal and abnormal biological processes.

Proposed Course of Project:

1) The minor polar lipids of Acanthamoeba will be identified. These probably include glycolipids and sulfolipids in addition to phospholipids. In particular there is a phospholipid of unknown composition which is the first compound to become labeled with $^{32}PO_4$ and attains the highest specific activity. It will be identified. Cells will be grown with a tracer dose of radioactive inositol to establish definitively the absence of inositol lipids.

2) Procedures will be developed for the direct isolation of the plasma membrane. Its composition and turnover will be compared to that of the membrane of the phagocytic vesicle which is derived from the plasma membrane. Somewhat larger molecules, such as phospholipids, will be used in these studies in the search for membrane precursors or alterations in membrane turnover.

3) Quantitative kinetic data will be obtained on the chemical and ezymatic changes which the phagocytic vesicles undergo during their lifetime in the cell by isolating the vesicles at different times during the phagocytic process. These experiments will be monitored at every stage by electron microscopy to correlate biochemistry and morphology.

4) The purification and characterization of the actin-like protein from platelets will be continued. These are model studies, on the one hand, for similar efforts to study the contractile protein of the amoebae. Attempts to isolate the protein from amoebae will be resumed as will efforts to relate its activity to phagocytosis.

5) Electron microscopy of the amoeba will be continued with special emphasis on membrane and fibrillar structures and the application of newer technqiues such as freeze-etching. Autoradiography will be expanded from the modest beginning made this year in order to apply it to studies of fatty acid transport in the amoeba.

Publications:

Korn, E.D. and Weisman, R.A., Phagocytosis of latex beads by Acanthamoeba II. Electron microscopic study of the initial events. Journal of Cell Biology 34, 219-227 (1967).

Korn, E.D., A chromatographic and spectrophotometric study of the products of the reaction of osmium tetroxide with unsaturated lipids. Journal of Cell Biology 34, 627-638 (1967).

Korn, E.D., Smith, F.R., and Weisman, R.A., Phagocytosis by an amoeba: a problem in membrane biochemistry. Protides of the Biological Fluids 15, 91-95 (1967).

Serial No. = NHI-24
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Physico-Chemical Studies of Fibrinogen

Previous Serial No.: None

Principal Investigators: Michael E. Friedman
Elemer Mihalyi

Man Years (computed for the 12 month period):
Total: 1.25
Professional: 1.25

Project Description:

Objectives: To determine the structure of fibrinogen

Methods Employed and Major Findings:
1) Ultraviolet spectroscopy and amino acid analyses of peptide residues; 2) Determination of molecular weight by ultracentrifugation; 3) Enzymology - trypsin digestion; 4) Peptide analysis - high voltage electrophoresis and descending chromatography; 5) Potentiometric titrations; 6) Purification methods - column chromatography; 7) Identification of subunits - electrophoresis on cellulose acetate strips; 8) Miscellaneous techniques - disc-gel electrophoresis, thin layer chromatography.

In previous work by Mihalyi and Godfrey it was observed that when bovine fibrinogen (mol. wt. approximately 350,000) is partially digested by trypsin (for 19 minutes) the molecular weight decreases to between 80,000 and 90,000. The digested material is homogeneous and retains many of the physical properties of the native material.

In the past year and one-half we have separated these fragments into three distinct species by column chromatography on DEAE sephadex and have analyzed their physical properties. All the fragments are very similar to one another in their chemical properties (e.g. amino acid analysis and fingerprints) and this indicates a high degree of symmetry within the molecule.

We then proceeded to cleave the disulfide bonds (by sulfitolysis) of the native protein and then separate the individual polypeptide chains by means of column chromatography using DEAE Sephadex.

Four peaks are observed from the chromatographic separation of the sulfitolyzed protein, and the molecular weight range is from 45000 to 60000. Previous work, from end group analysis, suggests that there are duplicates of three

different polypeptide chains (which makes a total of six individual chains). Physico-chemical (mol. wt) and chemical (amino acids and fingerprints), analysis of the polypeptide chains from each of the peaks indicates that the first two peaks are quite similar. This leads to a possible conclusion that the difference between the first two peaks was caused by the conditions under which the native fibrinogen was sulfitolyzed which might lead to changes relative to sulfhydryl groups or possibly changes in the carbohydrate moiety. However, this still does not preclude the fact that there could be minor chemical differences between the chains, and that the original assumption of three duplicate chains may be wrong.

Each of the tryptic fragments was sulfitolyzed and the molecular weights which were obtained ranged between 25000 and 35000. Since the molecular weight of the unsulfitolyzed fragment is in the neighborhood of 85000, this suggests that the tryptic fragments are composed of two to three chains.

Significance to Bio-Medical Research:
Some of the proteolytic fragments of fibrinogen are potent inhibitors of clotting and they may have a role in the control of intravascular clotting. Therefore, the mechanism of formation and the structure of these fragments may have some bearing on the problem of thrombo-embolic diseases.

Proposed Course of Project:
We shall try to identify the constituent polypeptide chains within each of the separated large fragments of fibrinogen by comparing the amino acid composition and the fingerprints (peptide maps) of the chains with those of the fragments.

Publications:
None.

Serial No. - NHI-25
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1968

Project Title: Alkali denaturation of fibrinogen

Previous Serial No.: None

Principal Investigator: Elemer Mihalyi

Man Years (computed for the 12 month period):

Total:	.75
Professional:	.75

Project Description:

Objectives: To correlate the unfolding of the molecule with a structural model proposed.

Methods Employed and Major Findings:
Ultraviolet spectroscopy, optical rotation measurements, fluorescence intensity measurements, etc.

Alkali denaturation of fibrinogen causes among other things:

1) An increase in the specific rotation; 2) an increase in the UV absorption in the region of the phenoxide ion abosrption; 3) an increase of the fluorescence intensity of a dimethylaminonaphthalene sulfon group coupled to the protein; and 4) loss of solubility at neutral pH. Kinetic studies have shown that the first three changes are simultaneous, whereas the loss of solubility occurs at a much faster rate than the change in optical properties. These reactions are all of the first order type and there is no evidence of more than one reaction class in the overall process. The results can be rationalized on the basis of the three beads model of the molecule. If the three beads unfold independently of each other, but with the same rate, there will be only one single reaction and the optical changes will be a reflection of the average unfolding of the whole molecule. On the other hand, if unfolding of a single bead in a molecule is sufficient to cause loss of solubility at neutral pH, then the rate of the latter will be much larger than that of the overall unfolding reaction. It is possible to calculate on statistical grounds the distribution of the molecules with none, one, two and three beads unfolded at any time of the reaction. The distribution curves can be compared with the experimental curves of average fraction unfolded and fraction which became insoluble. It turns out that the best fit is obtained with the assumption that the unfolding of a single bead is sufficient to cause insolubility. The

assumption that two or three unfolded beads are required for the change causes the precipitability curve to lag behind the overall curve over the first half or more of the reaction.

The rate of alkaline denaturation of fibrinogen was determined at various temperatures. From this an activation energy of 26000 cals per mole was calculated. This is lower than that of the thermal denaturation and is consistent with a gradual unfolding of the molecule under the stress of the repulsive forces brought about by the high charge on the polypeptide chains.

The rate increases markedly with pH. There is a straight line relationship between log rate and pH, with a slope close to unity.

Significance to Bio-Medical Research:
This study deals with the structure of fibrinogen, the central element in blood coagulation.

Proposed Course of Project:
Completed.

Publications:
None.

Serial No. - NHI-26
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigation of structural and functional relationships in proteins.

Previous Serial No.: NHI-236

Principal Investigator: Frederick H. White, Jr.

Other Investigator: Barbara Hauck

Man Years (computed for the 12 month period):

Total:	0.2
Professional:	0.1
Other:	0.1

Project Description:

Objectives: To investigate the mechanisms by which the secondary and tertiary structures are formed in proteins during biosynthesis. The observation (F.H. White, Jr., J. Biol. Chem. 239, 1032 (1964)) that introduction of 5-dimethylamino-1-naphthalene-sulfonyl (DNS) groups into ribonuclease (RNase) markedly diminishes its refolding ability was investigated further to establish which amino acid residues are attacked by DNS chloride, to give further insight as to which residues are involved in the refolding process.

Major Findings:
1. The following indirect evidence has been obtained to suggest that serine residues may be involved in the reaction between DNS chloride and proteins: (a) One fluorescent peptide has been isolated from DNS-lysozyme, which appears to be 31-39, or ala.ala.lys.phe.glu.ser.asn.phe.asn.; (b) Two major fluorescent peptides have been isolated from RNase, which are: 48-51 and 86-90; or his.glu.ser.leu. and glu.ser.thr.gly.ser., respectively; (c) The appearance of the glu.ser. combination is common to all three peptides, to suggest that this sequence may be involved in the reaction with DNS chloride; (d) A possible reaction mechanism would involve the attraction of the amino group of the DNS group to the carboxyl of the glutamyl residue, followed by reaction of the sulfonyl chloride moiety with the hydroxyl group of serine. The use of atomic models indicates that the spacing between the amino and sulfonyl chloride groups is nearly equal to that between the carboxyl and hydroxyl groups. Therefore the reaction is stereochemically feasible.

99

2. Repeated attempts by a variety of methods to isolate DNS derivatives of the amino acids in the isolated peptides or otherwise identify the DNS residues, have been unsuccessful. This project does not appear, at this juncture, promising for further investigation.

Significance to Bio-medical Research:
This work was undertaken to gain a deeper understanding of the biosynthesis, structure, and function of proteins, and in particular to investigate the factors involved in the formation of the native coiling and folding of the protein chain during its biosynthesis.

Proposed Course of Project:
Discontinued.

Publications:
None.

Serial No. - NHI-27
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Radiation damage in proteins

Previous Serial No.: NHI-237

Principal Investigators: Frederick H. White, Jr.
Peter Riesz (NCI)
Hideo Kon (NIAMD)

Other Investigators: Barbara Hauck (Technician)
Judith Clay (Technician, NCI)

Man years (computed for the 12 month period):

Total:	0.6
Professional:	0.3
Other:	0.3

Project description:

Objectives: To investigate the effects of radiation on the molecular structures and biological activities of proteins.

Methods Employed and Major Findings:
An original technique of carbon free-radical labeling (F.H. White, Jr., P. Riesz, and H. Kon, Radiation Res. 32, 744 (1967)) involving the use of free radical interceptors as labeling agents, has been employed for studying the carbon free-radical distributions in gamma-irradiated proteins.

Original procedures have been used for the reduction and carboxymethylation of proteins (F.H.White, Jr., Methods in Enzymology, Vol. XI, Academic Press, Inc., 481, 1967) and for the assay for enzymatic activity in ribonuclease (F.H. White, Jr., and A. Sandoval, Biochem. 1, 938 (1962)).

Findings:
1. The validity of the free-radical interceptor technique as a means for studying carbon free-radical distributions in irradiated proteins rests upon the following hypothesis: that the distribution of tritium is equivalent to the distribution of carbon free-radicals prior to exposure to HST. To test this hypothesis, tritium iodide was used as the free-radical interceptor in comparison to HST. The tritium distributions obtained with these two reagents were markedly similar for several proteins, indicating that the terminal reactions, which differ for these two reagents, do not distort the tritium distribution.

101

2. As a further test of this hypothesis, the irradiation and tritiation of gelatin and collagen were considered. These proteins incorporate tritium to a specific activity of approximately 30uC/mg. This value is about thirty times the values usually found for other proteins. It does not appear likely that the additional tritium is bound by any mechanism that would result in a distortion of the tritium distribution, since this distribution for gelatin does not differ appreciably from that found for other denatured proteins. However a study was undertaken to obtain more information about this mechanism and specifically to examine the possibility that proline or hydroxyproline residues, both of which are present in collagen and gelatin in high concentrations, may be responsible for the unusually high tritium uptake. Both ribonuclease and lysozyme have been tritiated in the presence of polyproline and polyhydroxyproline, with resulting tritium incorporations higher than those usually observed by factors of 2 to 4. It would therefore appear that those residues would account at least in part for the excessive tritium incorporation, and the investigation continues to determine the mechanism involved.

3. When native proteins are tritium-labeled by the free-radical interceptor method at room temperature, a characteristic tritium distribution is observed for each protein. However, when denatured proteins are labeled under identical conditions, a more nearly constant distribution is seen among the various proteins studied. Methods have been devised for the tritium-labeling of proteins which have been irradiated at the temperature of liquid nitrogen. The results indicate essentially the same distribution for native proteins as that obtained for denatured proteins at room temperature. This finding suggests a distribution of tritium which may be closer to the "primary" distribution than to the "secondary" distribution and that the "primary" distribution may not be influenced by conformation. (The "primary" distribution, as defined by Henriksen et al. (Radiation Res. 18, 147 (1963)), is that which results in a characteristic E.S.R. pattern when the protein is irradiated at the temperature of liquid nitrogen and examined by E.S.R. at this temperature. However, when the protein is warmed to room temperature after irradiation at the lower temperature and then examined by E.S.R., or when the sample is irradiated at room temperature and then so examined, another pattern results, which is characteristic of the "secondary" distribution. It is this distribution which is normally studied by the free-radical interceptor method.)

Significance to Bio-medical Research:
This work is of basic significance to the understanding of the nature of radiation damage to materials of biological origin. It is generally recognized that free radicals play an important role in radiation damage, and therefore emphasis has been placed on the study of their formation and location within the irradiated protein molecule.

Proposed Course of Project:
The investigation of factors that govern free-radical formation and distribution will be continued. The relationship between free-radical distribution and protein conformation will receive particular attention with emphasis on methods that are potentially useful for the selective labeling of

interior and exterior residues. It is anticipated that such a means for the differentiation between the surface and interior of a protein molecule would be useful in the study of protein conformation.

Publications:

P. Riesz and F.H. White, Jr., Advances in Chemistry Series, in press.
P. Riesz and F.H. White, Jr., Nature 216, 1208 (1967)
F.H. White, Jr., P. Riesz, and H. Kon, Radiation Res. 32, 744 (1967)

Serial No. - NHI-28
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Distribution of carbon free radicals among individual residues of gamma-irradiated dry lysozyme.

Previous Serial No.: None

Principal Investigator: Frederick H. White, Jr.

Other Investigator: Barbara Hauck

Man Years (computed for the 12 month period):

Total:	0.8
Professional:	0.4
Other:	0.4

Project Description:

Objectives: To investigate the distribution of carbon free-radicals, resulting from gamma-irradiation, among individual residues of an irradiated protein, for the purpose of confirming and extending the earlier observations that the free-radical distribution is a function of protein conformation.

Methods Employed and Major Findings:
The protein was tritiated by gamma-irradiation and exposure to tritiated hydrogen sulfide (HST). This procedure, "the free-radical interceptor technique", is an original method, described elsewhere (F.H. White, Jr., P. Riesz and H. Kon, Radiation Res. 32, 744 (1967)). The tritiated protein was reduced and carboxymethylated (F.H. White, Jr., Methods in Enzymology, Vol. XI, Academic Press, Inc., p. 481, 1967) to render it susceptible to digestion with chymotrypsin. The digestion and subsequent chromatography on phosphocellulose were performed according to Canfield and Anfinsen (J. Biol. Chem. 238, 2684 (1963)). After additional purification by high voltage paper electrophoresis, each peptide was hydrolyzed and then analyzed on an automatic amino acid analyzer in conjunction with scintillation flow counting to determine the specific activities of the individual residues in the lysozyme chain.

Findings:
1. The tritium distribution, as indicated by the specific activities of the individual residues, was widespread, with only twelve residues being either devoid of label or so weakly labeled that no activity could be

detected for them.

2. A wide variation in specific activity among amino acids of like species was observed, for example, among the six lysine residues, the values were: 1.78 (residue no. 1), 0.19 (no. 13), 0.79 (no. 33), 1.28 (nos. 96 and 97), and 0.69 (no. 116). These values are expressed as "relative specific activities" defined as: the ratio of the specific activity of a given residue to the average specific activity for the same kind of amino acid, derived from all of the peptide analyses.

3. The more highly labeled residues (relative specific activity = 1.5 or greater), when related to the three dimensional X-ray diffraction model of lysozyme, are found in several areas. Thus, residues 16, 18, 19, 21, 23, 29, 31, and 32 are heavily labeled and may be grouped with residues 105, 110, 113, and 115, also heavily labeled. Residues 50, 54, and 57 are similarly associated with 83, 84, and 88. At the amino end, residues 1 and 6 are heavily labeled, as are residues 118, 119, and 121 at the carboxyl end.

These results confirm previous indications (P. Riesz, F.H. White, Jr. and H. Kon, J. Am. Chem. Soc. 88, 872 (1966); F.H. White, Jr., P. Riesz, and H. Kon, Radiation Res. 32, 744 (1967)) that the conformation of the protein molecule influences the tritium distribution and therefore the carbon free-radical distribution of the irradiated protein.

Significance to Bio-medical Research:
This work is of significance to the understanding of the nature of radiation damage to materials of biological origin. It is generally recognized that free radicals play an important role in radiation damage, and therefore emphasis has been placed on the study of their formation and location within the irradiated protein molecule.

Proposed Course of Project:
Concluded.

Publications:
F. H. White, Jr., Radiation Res., submitted for publication.

Serial No. - NHI-29
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigation of the free-radical interceptor technique as a means for the preparation of tritiated proteins.

Previous Serial No.: None

Principal Investigators: Frederick H. White, Jr.
Peter Riesz (NCI)

Other investigators: Barbara Hauck (Technician)
Judith Clay (Technician, NCI)

Man Years (computed for the 12 month period):

Total:	0.4
Professional:	0.2
Other:	0.2

Project Description:

Objectives: To investigate the possibility of employing the free-radical interceptor technique (F.H. White, Jr., P. Riesz, and H. Kon, Radiation Res. 32, 733 (1967)) as a means for the tritiation of proteins.

Methods Employed and Major Findings:
Tritium labeling of proteins was achieved by the method of free-radical interception, which involves gamma-irradiation (Co^{60}) of a lyophilized protein in vacuo, followed by exposure to tritiated hydrogen sulfide (HST) until all of the free radicals (produced by irradiation) have disappeared, as indicated by the disappearance of the E.S.R. spectrum.

Findings:
1. When tritiated ribonuclease (T-RNase) and tritiated lyoszyme (T-lysozyme) were chromatographed on columns of XE-64, and their behavior was similar to that of the corresponding native proteins, with nearly all of the radioactivity moving with the "native" peaks.

2. Repeated crystallization of T-lysozyme indicated the loss only of an amount of tritium which was attached to the exchangeable sites, suggesting the absence of radiolytic breakdown products.

3. The enzymatic activities of T-RNase and T-lysozyme were not significantly affected by the tritiation procedure.

. The possibility of racemization (by free-radical formation) of the alpha arbon atoms of amino acids was investigated by hydrolysis of the tritiated roteins, followed by digestion with D-amino acid oxidase and amino acid nalysis (with scintillation flow counting). Thus far, there has been no ndication that the specific activities of the amino acids for which this nzyme is specific are any lower than those of the control experiment carried out identically in the absence of D amino acid oxidase). Therefore here is as yet no evidence for any contribution by racemization to eterogeneity of the tritiated protein.

. With the use of H_2S^{35} in place of HST, it has been shown that the protein raction (from XE-64 chromatography) which incorporated sulfur is easily eparable and contains no significant amount of tritium.

ignificance to Bio-medical Research: Tritiated proteins could be of use s substrates or precursors in a large number of biological investigations. he method of free-radical interception would have an advantage over the ommonly used external labeling methods (e.g. iodination) in that the sotope is broadly distributed, with nearly every amino acid being affected, ermitting the fates of all parts of the protein chain to be studied, ithout introduction of labeling groups that are foreign to the protein and hich might therefore have subtle effects upon the protein conformation. lthough tritiation of proteins has been tried with the use of tritium as (D. Steinberg, et al., in Liquid Scintillation Counting, p. 230, ergamon Press, N. Y. (1958), the relatively high yields of apparently omogeneous tritiated protein obtained from the free-radical interceptor ethod suggest that HST may be the reagent of choice, having the advantage hat approximately one hundredth as much gaseous radioactivity is involved, ith correspondingly less radiation hazard and radiolytic breakdown of rotein.

roposed Course of Project:
ther proteins will be investigated by tritiation with the free-radical nterceptor method, to be followed by examination by a variety of methods o establish the extent of homogeneity of the tritiated products. Experi- ents will be undertaken to increase the extent of labeling of the various roteins studied.

ublications:
. H. White, Jr. and P. Riesz, Biochem. and Biophys. Res. Comm. 30, 303 1968).

Serial No. - NHI-30
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Protein Synthetic Capabilities of Membrane Fragments

Previous Serial No.: NHI-240

Principal Investigators: Richard Hendler
Raymond Scharff

Collaborating Investigator for Electron Microscopic Studies:
N. Nanninga of the Laboratory for Electron Microscopie, University of Amsterdam, Netherlands

Man Years (computed for the 12 month period):

Total:	2
Professional:	2

Project Description:

Objectives: We are approaching this problem from two directions that we hope will eventually converge. This report concerns the studies of protein synthetic capabilities of E. coli membrane fragments. The accompanying report describes studies on the biological oxidative capabilities of corresponding membrane fractions. The primary source of energy in a respiring cell comes from a series of discrete oxidation reactions called electron transport. These reactions occur in cell membranes. My work in protein synthesis, as well as that of several other groups, has provided strong evidence that the primary site of protein synthesis is at a membrane surface. Current ideas in protein synthesis suggest that energy produced in a membrane from electron transport is sent out into the aqueous environment as ATP and eventually brought back to the membrane in the form of activated amino acids. We are interested in the possibility that these two major metabolic pathways, one exergonic and the other endergonic, may be directly coupled in the membrane.

Methods Employed and Major Findings:
Preparation of spheroplasts and membrane fragments.

By visible and viable cell counts and phase contrast microscopy it was determined that during the course of production, isolation, washing and storage in liquid nitrogen of penicillin-produced spheroplasts, 95% of the starting cells were broken. Another 2% of the cells could be broken by

15 seconds ultrasonication. Since we are attempting to obtain membrane fragments with the least amount of physical damage the ultrasonication step has been eliminated. Centrifugation at 3500 x g for 10 minutes sediments 99% of the residual whole cells and the largest sized membrane fraction. This is the P_I fraction. Centrifugation of the P_I supernatant at 20,000 x g for 15 minutes sediments the remaining whole cells and a large number of small membrane fragments which constitutes the P_{II} fraction. The P_{II} supernatant (S_{II}) contains the free ribosomes, soluble fraction and small sized membrane fragments. The particulate components can be isolated by centrifugation at 100,000 x g for 60 minutes (P_{III} fraction). The small membrane vesicles can be separated from free ribosomes by sucrose density gradient centrifugation. A different method of isolation starts with a 20,000 x g, 15 minute pellet (P_I & P_{II}). This pellet is placed on top of a double layer of sucrose, 80% on the bottom and 50% on top. Centrifugation for 30 minutes in centrifugal fields from 18,000 to 130,000 x g causes most of the membrane material (determined by phospholipid, protein and NADH oxidase activity) to sediment to the interface. A variable number of residual whole cells will penetrate through the 80% sucrose. This preparation of membranes is called "interface material".

Electron microscopy of membrane (or envelope) fractions:
The P_I fraction consists mainly of large envelope fragments (about 500 mμ) which appear either as ovals or open spirals. Some smaller closed vesicles are also encountered. Electron-dense granules which have the appearance and size of ribosomes are found lining the inner surface of most of these fragments. Whole cells are occasionally seen.

The P_{II} fraction contains smaller vesicles (200-500mμ) most of which are closed and many of which are found as vesicles within vesicles. Electron-dense ribosomal-type granules are also found along the inner surface of these profiles. The interface materials appears similar to the P_I and P_{II} fractions.

Chemical composition of membrane fragments:
The bulk of the dry weight of E. coli envelopes is made up of protein, lipid and RNA. Whole cells consist of approximately 63% protein, 24% RNA and 13% phospholipid. From sonicated spheroplasts, P_I contains respectively 58%, 14%, 28% and P_{II} contains 67%, 5%, 28% protein, RNA and lipid. Combined P_I and P_{II} from unsonicated cells contains 50%, 22% and 28%, protein, RNA and lipid; whereas the interface material contains 43%, 30%, and 27%. The value of 30% RNA for the interface preparation may be compared to values of 1-9% reported in the literature by investigators who used more drastic procedures to obtain membranes. Our higher values for RNA content indicate that we are obtaining fragments with more attached ribosomes.

Incorporation of amino acids by membrane fractions:
Since some residual whole cells remain in our membrane fractions it is most important to establish that the observed amino acid incorporation is not entirely due to the whole cells. The proportion of whole cells to membrane fragments is greater in P_I than in P_{II}. In order to assess

the relative amino acid incorporating activities of membrane fragments and whole cells, all data is considered in terms of total amino acid incorporation of the fraction divided by the total number of whole or viable cells present. Equal values for P_I and P_{II} would indicate that the membrane fragments are relatively inert. On the other hand if membrane fragments contribute significantly to the incorporation ability then incorporation per viable cell in P_{II} should be higher than incorporation per viable cell in P_I. Experimental values of about two are usually encountered. This advantage of P_{II} over P_I is enhanced by providing a complete mixture of amino acids and an ATP generating system. A synthetic medium instead of a soluble cell extract also benefits P_{II} more than P_I.

When μmoles of amino acid incorporated per mg of RNA is calculated for P_{II} and compared to published values obtained with E. coli ribosomal systems, it is found that the activity of the P_{II} fraction is generally several-fold higher than the activities obtained in fully primed ribosomal systems which are stimulated by the addition of polynucleotide messenger RNA. Therefore, without the addition of exogenous messenger, the ribosomes in our preparation appear to be operating at maximal efficiency.

Significance to Bio-Medical Research:
This project is designed to provide some understanding of the manner in which an organized cellular system may efficiently integrate two complex and dependent metabolic pathways. The potential importance of biochemistry lies in working out chemical pathways and then considering this knowledge in terms of living cells and tissues. If it could be demonstrated that the structural organization of cell contributes to its ability to integrate complex and related biochemical pathways, a better understanding of cellular function would result.

Proposed Course of Project:
1. Heavy membrane fragments and residual whole cells sediment to the bottom of a 50% sucrose solution in centrifugal fields greater than 18000 x g. Whole cells have a tendency to penetrate an 80% sucrose solution but membrane fragments do not. Further separation of heavy membrane fragments from residual intact cells will be attempted by centrifugation in sucrose solutions between 50% and 80% concentration.

2. A microscopic radioautographic procedure will be applied to the cleanest membrane preparations in order to determine directly the extent of amino acid incorporating ability due to whole cells and that due to acellular fragments.

3. Specific enzyme treatments followed by electron microscopy will be used to define the nature of ribosomal-membrane associations.

Publications:
Hendler, R.W., Protein Synthesis as a Membrane-Oriented Cellular Activity, Protides of the Biological Fluids, 15, 37 (1967).

Serial No. - NHI-31
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Biological oxidation capabilities of membrane fragments obtained from E. coli.

Previous serial No.: NHI-240a

Principal Investigator: Richard W. Hendler

Other Investigator: Amelia H. Burgess

Man Years (computed for the 12 month period):

Total:	1.8
Professional:	.8
Other:	1.0

Project Description:

Objectives: The primary source of energy in a respiring cells comes from a series of discrete oxidation reactions called electron transport. These reactions occur in cell membranes. My work in protein synthesis, as well as that of several other groups, has provided strong evidence that the primary site of protein synthesis is at a membrane surface. Current ideas in protein synthesis suggest that energy produced in a membrane from electron transport is sent out into the aqueous milieu as ATP and eventually brought back to the membrane in the form of activated amino acids. We are interested in the possibility that these two major metabolic pathways, one exergonic and the other endergonic, may be directly coupled in the membrane. We are approaching this problem from two directions that we hope will eventually converge. This report concerns the studies of biological oxidation in E. coli membrane fragments. The accompanying report describes studies on the protein synthetic capabilities of corresponding membrane fractions.

Methods Employed and Major Findings:

Preparation of spheroplasts and membrane fragments:
The technique used to produce spheroplasts from cells growing on glucose did not produce spheroplasts from cells growing on succinate or malate. Conditions were worked out which allowed the efficient production of spheroplasts from cells actively oxidizing these Krebs cycle intermediates. Membrane fractions corresponding to P_I, P_{II}, and P_{III} described in the accompanying report on protein synthesis were prepared. In addition, a sonicate of whole cells (S_I) and a supernatant fraction (S_3) obtained by

111

centrifugation of S_1 at 105000 x g for 60 minutes were prepared.

Distribution of dehydrogenase and oxidase abilities in fractions obtained from E. coli and the influence of previous growth history of the cells on these activities:
Dehydrogenase activity was measured with an automatic spectrophotometer by the change in state of reduction of the dye 2.6 dichlorophenol indophenol. Oxidase was measured with the aid of an oxygen electrode or by the oxidation of NADH or NADPH monitored spectrophotometrically.

Oxidative capability for all fractions from cells grown with the three different carbon sources (glucose, malate, succinate) were tested against the following substrates: NADH, NADPH, succinate, malate, isocitrate, glutamate, pyruvate, α-ketoglutarate.

It was found that: (1) There is a very active membrane-bound NADH-oxidase system; (2) The conventional ribosome fractions (100000 x g pellet from 20000 x g supernatant fraction) contains small-sized membrane vesicles possessing very high NADH-oxidase and succinoxidase activity; (3) These small vesicles ($\leq$ 200mμ in diameter) contain a granular substance (approximately 80Å in diameter) along their inner surface and they can be separated from free ribosomes by centrifugation in a sucrose gradient. The vesicles contain all of the oxidase activity of the ribosome fraction; (4) The oxidase ability of these systems is sensitive to cyanide, but not to azide, rotenone, antimycin A, oligomycin, ICI 47776, the availability of ADP, or 2,4 dinitrophenol. Therefore a marked contrast to conventional mitochondrial systems is apparent; (5) Malate dehydrogenase is present, soluble and requires NAD. Isocitrate dehydrogenase is present, soluble and requires NADP. But although the oxidation of NADH and NADPH in these preparations is readily coupled to oxygen uptake, the dehydrogenation of malate and isocitrate is not efficiently linked to oxygen uptake. Complexes of the dehydrogenases with their respective co-enzyme are therefore indicated, although alternative explanations are not discounted; (6) The functioning of an intact Krebs oxidative cycle is questioned because although growth in succinate increases succinoxidase; malate or isocitrate dehydrogenases and oxidases are not increased. Furthermore, growth in malate does not increase oxidative capacity for malate, succinate or isocitrate.

Significance to Bio-Medical Research:
This project is designed to provide some understanding of the manner in which an organized cellular system may efficiently integrate two complex and dependent metabolic pathways. The potential importance of biochemistry lies in working out chemical pathways and then considering this knowledge in terms of living cells and tissues. If it could be demonstrated that the structural organization of cell contributes to its ability to integrate complex and related biochemical pathways, a better understanding of cellular function would result.

ANNUAL REPORT
CARDIOLOGY BRANCH, NHI
July 1967 to June 1968

The major long range objective of the research program of the Cardiology Branch, NHI, has been to develop an increased understanding of the derangements in cardiac function which occur in various forms of heart disease. In order to reach this objective, it has been appreciated that while investigations on man are extremely important, perhaps of greater significance are observations on isolated cardiac muscle and on animal models, since the pertinent hemodynamic and biochemical variables are so difficult to control in intact man.

Major personnel changes occurred in the Cardiology Branch during the period covered by this report. Fortunately, the research output of the Branch was largely unimpaired by these events. Indeed, many investigations which had been begun several years earlier were completed during this year in advance of the departure of several investigators.

The investigations carried out will be summarized under the following headings:

I. The Determinants of Myocardial Energy Requirements

II. Studies on the Contraction of the Intact Canine Heart

III. Investigations on Heart Failure

IV. Characterization of Myocardial Function in Man

V. Pharmacologic Investigations

VI. Studies on Patients with Congenital and Acquired Heart Disease

VII. Studies on the Peripheral Circulation

VIII. Other Physiologic Investigations

114

SECTION I

THE DETERMINANTS OF MYOCARDIAL ENERGY REQUIREMENTS

A. Investigations on Isolated Heart Muscle.

Although isolated cat papillary muscles have proved useful in a variety of physiologic and pharmacologic studies, the energetic integrity of this preparation has been questioned. Accordingly, papillary muscles (avg. diam. = 1.3 mm) were equilibrated in oxygenated Krebs solution for 1 hr at 26°C at rest or contracting at various frequencies and temperatures and for various durations. Measurements of high energy phosphate stores in the muscles were carried out and the results compared with those in right ventricles studies in vivo. It is concluded that papillary muscles even of moderately large diameter are energetically intact for at least 3 hr at frequencies of 12/min at 26°C. Further, energy stores are even greater than those found in vivo. However, at frequencies of 30 or 60/min energy stores may be limited.

An effort was made to define the chemical energetics of cardiac muscle by measuring the utilization of ATP and creatine phosphate (CP) in the papillary muscle of cat heart. To accomplish this, cat right ventricular papillary muscles were metabolically inhibited with iodoacetic acid and nitrogen to prevent further production of ATP and CP. The utilization of CP and ATP by isometrically contracting muscles could be accounted for by a prediction equation with terms for the number of activations and the calculated contractile element work. Other metabolically inhibited muscles were stimulated to contract isotonically. The efficiency of energy utilization for the performance of internal contractile element work was found to average 0.0067 μmoles~ P/g-cm of work and for external work was 0.0031 μmoles~P/g-cm, providing a demonstration of the Fenn effect in cardiac muscle. On the basis of other studies utilizing an identical experimental approach it was concluded that the "oxygen wasting" effect of norepinephrine on heart muscle may result from the increased utilization of energy associated with an increased contractile state. A study was also designed to determine whether a defect in energy utilization could be the fundamental defect in the congestive heart failure state. It was concluded that in heart failure the net utilization of~P is reduced, but only in relation to the reduction in contractile element work, and that the direct conversion of chemical energy to mechanical work is not an inefficient process in this state.

Certain features of clinical and experimental hyperthyroidism have been considered manifestations of changes in chemical energy generation. Alterations in efficiency of energy utilization may also be important in hyperthyroidism, but this efficiency has not been measured directly. Accordingly, conversion of chemical energy to mechanical work was compared in myocardium obtained from hyperthyroid and normal cats. The utilization of chemical energy, consisting of creatine phosphate and ATP, was determined in isolated right ventricular papillary muscles treated with iodoacetic acid and nitrogen, as described above. The basal rate of energy utilization in muscles resting without tension was higher in 39 muscles from

1.C

hyperthyroid animals than in 76 normal muscles (0.99 vs. 0.77 μmoles Δ P/g/min). It was also observed that hyperthyroid muscles utilized significantly more energy to perform a given amount of work than did normal muscles. This inefficiency in conversion of chemical energy to mechanical work in hyperthyroid myocardium implies an action of thyroxin on the mechano-chemical coupling process during cardiac muscle contraction.

The oxygen consumption of isolated papillary muscles from normal and hyperthyroid cats was also studied during isometric and isotonic contractions using a polarographic oxygen electrode. At equivalent levels of tension development muscles from hyperthyroid cats had a higher oxygen consumption than the normal group. These results are compatible with the premise that augmentation of contractile state is associated with an increase in myocardial oxygen consumption.

The development of tension has long been recognized as an important determinant of energy utilization of both skeletal and cardiac muscle. In addition, Fenn demonstrated in skeletal muscle that the onset of shortening against a load results in an additional energy utilization which is proportional to the work done. The applicability of these observations to the myocardium have not been clear. Accordingly, the effects of tension development and external work on the energy utilization of heart muscle were investigated by measuring both the mechanical performance and oxygen consumption of papillary muscles with a polarographic technique. The relative effects of tension and external work were determined by comparison of O_2 consumption of isometric contractions to the O_2 consumption of afterloaded contractions at identical levels of tension development. The performance of external work in afterloaded contractions was found to be associated with an additional energy utilization which was directly proportional to the external work performed. The unit energy cost of performing external work was found to be about one-half the unit energy cost associated with "internal work" performed by the contractile elements in stretching the series elastic components. These data indicate that in addition to the development of tension and the velocity of contraction, external work is a mechanical correlate of energy utilization in heart muscle, as it is in skeletal muscle.

The relative importance of tension maintenance and tension development in determining the oxygen consumption of cat papillary muscle in vitro was also studied by comparing the amount of oxygen used during normal isometric contractions with that utilized during isometric contractions which are quick-released to preload tension during the course of contraction. It appears that maintenance of tension adds relatively little to the oxygen cost of tension development, indicating that compared to tension development, tension maintenance plays a relatively minor role in determining oxygen consumption.

The effects of norepinephrine on the mechanical performance and oxygen consumption were studied in the cat papillary muscle preparation using a polarographic technique. Norepinephrine augmented the contractile state of both isotonic and isometric contractions as well as the myocardial oxygen consumption. At a constant level of Tension development, increments in oxygen consumption were found to be directly proportional to the degree

of augmentation of the contractile state. These data, like those obtained from the muscles in which creatine phosphate and ATP utilization were studied, suggest that increased myocardial O_2 consumption following norepinephrine is directly related to changes in the contractile state and not due to an "oxygen wasting effect." Similarly, increasing the frequency of stimulation or the Ca^{++} ion concentration resulted in augmentation of both the velocity of contraction and the extent of shortening and work done. These changes in mechanics at a constant level of tension development were associated with increased myocardial oxygen consumption. These findings lend further support to the view that the contractile state of heart muscle is an important determinant of oxygen consumption.

B. Investigation on the Intact, Canine Heart.

An investigation was carried out in order to provide a direct comparison of the quantitative effects of tension development and contractile state on myocardial oxygen consumption ($M\dot{V}O_2$). This was accomplished in the isovolumetrically contracting canine left ventricle by varying peak developed tension (PDT) at a constant level of contractility, and conversely by varying contractility at a constant PDT in the same animal. $M\dot{V}O_2$ consistently increased with changes in both PDT and contractile state. Multiple regression analysis yielded the following equation: MVO_2 (μl/beat/100 g LV) = -35 + .25 PDT (g/cm^2) + 1.43 V_{max} (cm/sec). It was concluded that the oxygen cost of alterations in contractile state (V_{max}) of the intact heart is substantial and of an order of magnitude similar to that associated with alterations in myocardial wall tension.

The relationship between heart rate and myocardial oxygen consumption ($M\dot{V}O_2$) was studied in 8 canine isovolumic left ventricles while controlling peak wall stress. Increasing rate was found to increase the contractile state of the heart and also to increase the $M\dot{V}O_2$ per beat. These results are in agreement with previous experiments showing a direct relationship between contractile state of the heart and $M\dot{V}O_2$.

The effects of acute simulated mitral and aortic regurgitation on myocardial oxygen consumption were then examined. Despite large increases in total stroke volume and the extent of fiber shortening, oxygen consumption increased only moderately (14% with MI) (17% with AI). Since, as re-emphasized by this investigation, the energy expended by the contractile elements in shortening the myocardial fibers is very small relative to the energy cost of stretching the series elastic component, i.e. developing tension, the total energy costs of acutely induced mitral and aortic valvular regurgitation are relatively modest, and can be explained in part by the increases in tension which are induced.

Previous studies concerning the effects of counterpulsation at $M\dot{V}O_2$ have suggested that the fall in $M\dot{V}O_2$ is relatively less than would be predicted from pressure-time indices. Since $M\dot{V}O_2$ is more closely related to myocardial wall stress than to pressure, the effects of counterpulsation on the mechanics of left ventricular contraction and $M\dot{V}O_2$ were examined in 9 dogs utilizing synchronized counterpulsation through the abdominal aorta. Heart rate was controlled and cardiac output was maintained constant by

right heart bypass.

With counterpulsation, decreases were observed in peak LV wall stress, integrated wall stress, contractile element work, and fiber shortening work, while the extent of fiber shortening increased. Coronary blood flow increased and tended to remain elevated after counterpulsation; myocardial oxygen consumption decreased significantly. These results support the view that when the myocardial contractile state is constant, $M\dot{V}O_2$ is determined by wall stress and the work of the contractile fibers.

The effects of increased cardiac workload on myocardial high-energy phosphate stores were examined in 6 dogs utilizing an isovolumically contracting left ventricular preparation. During control periods there was significantly more epicardial than endocardial creatine phosphate (CP). With increased cardiac workload secondary to an increase in heart rate and volume there was a slight fall in epicardial CP, but no changes in endocardial CP or in the levels of ATP. Thus, in this preparation there is a normal transmural gradient of CP and marked increase in wall stress, and heart rate can be sustained with only minor alterations of the heart's total energy stores, and of the normal transmural gradient.

SECTION II

STUDIES ON THE CONTRACTION OF THE INTACT CANINE HEART

The gross architecture and ultrastructure of the contracting left ventricle of the dog had been analyzed in the past, but little information concerning the spatial orientation of the muscle fibers during diastole and systole has been available. To obtain this, full thickness specimens, extending from base to apex and across the left ventricular free wall, were obtained in 18 dogs' hearts rapidly fixed in situ, arrested in diastole, systole, and in dilated diastole. Fiber orientation was determined across the wall in serial sections. The angle of the circumferential fibers relative to a horizontal reference plane perpendicular to the left ventricular major axis exhibited a transition from an average of -62.0° at the epicardium, through 0° at midwall, to +62.1° at the endocardium. When this angle was plotted against wall thickness, the resulting curvilinear relations, averaged for all sampling sites, were not different statistically in the three groups of hearts. These findings indicate that the left ventricular wall is comprised of a nonlayered continuum of fibers and that average fiber orientation does not change significantly during contraction. The data should facilitate engineering analysis of left ventricular wall stress during the cardiac cycle.

When contractile state is constant, the two principal determinants of the velocity of myocardial fiber shortening are myocardial wall tension (afterload) and end-diastolic volume (end-diastolic length). Under controlled conditions, velocity of circumferential fiber shortening (V_{CF}), is inversely related to wall tension and directly related to instantaneous fiber length, but the effects of varying end-diastolic volume on V_{CF} and tension in normal intact closed chest dogs has not been known. The effect of varying end-diastolic volume on V_{CF} was therefore studied in normal closed chest dogs. It was observed that left ventricular wall tension is significantly higher throughout beats beginning at higher end-diastolic volumes. V_{CF} is inversely proportional to left ventricular wall tension, and this effect seems to counteract the expected influence of fiber length in increasing V_{CF}. The data suggests that V_{CF} may be unchanged or actually reduced by an increase in end-diastolic volume.

The intrinsic responses of the heart to an acute outflow resistance consist of an increase, followed by a small decrease in end-diastolic volume and pressure, a response termed homeometric autoregulation. It has been suggested that this response is associated with a very striking increase in contractility. In order to examine this question homeometric and heterometric (increase in end-diastolic volume) autoregulation were produced in the in situ dog left ventricle, in which beta adrenergic receptors had been blocked with propranolol, by increasing aortic pressure and stroke volume respectively. Effects of instantaneous fiber length were controlled by comparing single isovolumic beats originating from similar end-diastolic circumferences, measured with a mercury-in-rubber gage.

It was found that the increase in myocardial performance during an acute increase in left ventricular outflow resistance is principally a result

of the increased fiber length, with only a small additional increase in contractile state being attributed to homeometric autoregulation.

Recent studies which have applied isolated muscle mechanics to the intact heart have demonstrated that the overall performance of the ventricle is affected by its instantaneous loading. It has been shown that ventricular wall tension and the velocity of ejection are determined by the instantaneous impedance to ejection and ventricular size during ejection. The decrease in aortic compliance which accompanied aging in man increases the instantaneous impedance to ejection throughout systole and therefore results in an increased load. It was therefore of considerable interest to study the effects of increased aortic rigidity on left ventricular dynamics. This was accomplished in eight dogs by diverting their aortic blood flow through a rigid bypass. Despite unchanged contractility direction of blood through the bypass caused increases in peak systolic pressure, myocardial wall tension, the duration of ejection and left ventricular end-diastolic pressure. Thus this investigation illustrates that the behavior of the myocardium is conditioned in an important manner by the physical characteristics of the arterial tree.

In isolated muscle, the series elastic component (SEC) can be analyzed by determining the length changes following quick releases to known loads during contraction. However, no information on this important component in the intact heart has been available. Accordingly, the characteristics of the effective SEC of the intact left ventricle (LV) were determined by a quick release method in 11 dogs in which the LV contracted isovolumically against a balloon inserted through the mitral annulus. The load-extension curves obtained in this manner were found to be exponential and qualitatively similar to those obtained in isolated cat papillary muscle. They were unchanged by varying the time of release and by norepinephrine infusion.

In order to extend to the intact ventricle concepts of myocardial mechanics initially developed in isolated papillary muscles, left ventricular volume-tension relations of ejecting and isovolumic contractions were studied in closed-chest conscious, though sedated dogs. Isovolumic contractions were produced by sudden balloon occlusion of the ascending aorta. Instantaneous ventricular volume in ejecting beats was derived from the time integral of ascending aortic blood flow monitored by an implanted electromagnetic probe, and left ventricular end-diastolic volume was obtained from the pressure-volume relation of the passive ventricle. It was observed that the ejecting beats proceeded to a volume-tension relation at end-ejection which approximated within 3 ml. the volume-tension relation at the peak of isovolumic contractions. This finding indicates that the behavior of the intact ventricle resembles that of isolated cardiac muscle in that the relation between length and tension of maximum contraction is largely independent of initial muscle length.

The mechanical properties of left ventricular contraction were described in terms of tension, velocity, length and time in closed-chest, sedated dogs in which a large aorto-caval fistula had resulted in circulatory congestion. The results were then compared with those obtained in normal dogs. Instantaneous contractile element velocity was calculated from left ventricular pressure

and its first derivative during isovolumic left ventricular contractions produced by sudden balloon occlusion of the ascending aorta during diastole. A range of ventricular end-diastolic volumes was induced and heart rate was controlled. Wall tension (stress) was derived from ventricular pressure and volume, the latter being obtained from the pressure-volume relation of the passive ventricle.

It was observed that extrapolated velocity at zero tension, V_{max}, averaged 3.0 circ/sec in the normal dogs and 2.9 circ/sec in the seven dogs with an aorto-caval fistula and fluid retention. Isovolumic tension (P_o) in dogs with aorto-caval fistulae tended to be slightly greater than normal. Therefore, despite circulatory congestion, the ventricular contractile state usually was normal in the dog with a large aorto-caval fistula.

The mechanical properties of left ventricular contraction were also described in terms of tension, velocity, length and time in closed-chest, sedated normal, hypothyroid and hyperthyroid dogs. The hypothyroid state was associated with a displacement of the tension-velocity relation of the left ventricle downwards and to the left, i.e., a reduction in fiber shortening at any given level of tension and vice versa. In hyperthyroid state, the tension-velocity relation of the left ventricle was displaced upwards and to the right with a marked increase in V_{max} and a less prominent increase in P_o. The changes in the tension-velocity relations indicate that the inotropic state of the left ventricle in the intact dog varies directly with the animal's thyroid state.

Previous studies on the alterations in cardiovascular dynamics produced by valvular regurgitation have illustrated how these lesions affect the heart as a pump but have not described the ways in which fundamental myocardial muscle function is altered by the abnormal load. Accordingly, in 17 dogs, controlled, metered aortic and/or mitral valvular regurgitation was induced. With both lesions immediate increases occurred in total stroke volume, the extent of fiber shortening, end-diastolic pressure and peak wall tension. Furthermore, the rate of tension decline during ejection was augmented as was peak contractile element velocity. The extent of those latter increases was seen to result from lowered instantaneous impedance to ejection.

Thus, since the velocity and extent of contractile element shortening and therefore stroke work, ejection fraction, peak aortic flow, and stroke volume are dependent on the instantaneous load faced by the ventricle, the circulatory changes in valvular regurgitation can be explained by a consideration of the effects of these lesions on the impedance to left ventricular ejection.

As an outgrowth of this investigation the effects of left-to-right shunts on left ventricular contraction was considered. With equal left-to right shunts cardiocirculatory effects of patent ductus arteriosus (PDA) and ventricular septal defects (VSD) have generally been considered to be similar. In order to determine whether or not this is the case, the effects of acute, controlled, and metered PDA and VSD were studied in dogs by the use of external shunts. Left ventricular performance was compromised to a greater extent by PDA than VSD with equal shunts. This difference is

explained by the fact that in PDA the left ventricle must shunt the entire stroke volume into the high pressure aorta, while in VSD a large portion of this volume is shunted directly into the low pressure right ventricle.

SECTION III

INVESTIGATIONS ON HEART FAILURE

The intrinsic mechanical properties of isolated papillary muscles from hypertrophied and failing hearts have recently been defined. However, the level of mechanical function per unit of muscle within the intact but chronically hypertrophied and failing ventricle had not been defined previously.

Right ventricular (RV) hypertrophy and right heart failure were induced in cats by graded constriction of the main pulmonary artery. After hypertrophy and heart failure had been established for four weeks, right heart and aortic pressures were determined by catheterization and cardiac outputs and heart rate were measured. Following this, the RV pressure and rate of rise of pressure (dp/dt) were determined during an isovolumic beat produced by sudden occlusion of the pulmonary artery during the preceding diastole; the right atrium was opened, an RV papillary muscle was removed for study and the passive pressure-volume relations of the RV were determined. This allowed calculation of the maximum contractile element velocity of shortening and the tension developed per unit of cardiac muscle. For comparison, similar studies were done in eight normal cats.

In intact failing hearts, average RV weight, peak isovolumic pressure, RV end-diastolic pressure, RV end-diastolic volume, and maximum isometric tension (P_o) all increased significantly. However, the maximum extrapolated contractile element velocity (V_{max}) was markedly reduced; in the papillary muscles from the same ventricles, P_o at the apex of the length-tension curve and V_{max} were greatly reduced.

Thus, it was found that in heart failure, both force and velocity were depressed in the isolated muscle, while increased fiber length maintained force developed per unit of muscle in the intact ventricle. This increased preload and the greater muscle mass provided augmented total ventricular force and pressure development. However, V_{max}, which is unaltered by muscle length or mass, provided an accurate reflection of the depressed ventricular contractile state.

These findings provide a quantitative analysis of depressed intrinsic contractile state in the intact, chronically hypertrophied and failing myocardium indicating that the rate of interaction of contractile sites may be reduced in both hypertrophy and overt heart failure. They further demonstrate the manner in which augmentation of end-diastolic volume and total muscle mass compensates for this intrinsic defect.

Intensive investigation of the syndrome of chronic congestive heart failure (CHF) has resulted in delineation of alterations in function of organ systems, the autonomic nervous system, and the peripheral circulation. However, limitations imposed by assessing cardiac function in hemodynamic terms have, in the past, made it difficult to define the contractile state during CHF.

The myocardial contractile state and energy stores were evaluated in congestive heart failure produced in dogs by chronic ventricular stimulation

at 280 contractions per minute. Twenty-four hours following cessation of stimulation, analysis of the mechanics of isovolumic left ventricular contractions revealed marked depression of the contractile state. This syndrome of heart failure was also associated with significant reduction in total myocardial energy stores. The findings demonstrate that heart failure following chronic tachycardia is associated with a depressed contractile state in the absence of evidence of significant left ventricular hypertrophy.

Although previous studies have suggested a depression of myofibrillar ATPase in the presence of heart failure, their interpretation has been limited by failure to exclude mitochondrial contamination and lack of information regarding the contractility of the tissue studied. To explore this fundamental question, myofibrils were prepared from the right and left ventricles of normal cats, cats with right ventricular hypertrophy and with right ventricular hypertrophy and right ventricular failure. In right ventricular failure, right ventricular myofibrillar ATPase was found to be depressed. However, in right ventricular hypertrophy, right ventricular myofibrillar ATPase was not significantly depressed. The contractility of the associated right ventricular papillary muscles, expressed as maximum rate of force development at the apex of the length-active tension curve, was found to be correlated with the myofibrillar ATPase activity.

SECTION IV

CHARACTERIZATION OF MYOCARDIAL FUNCTION IN MAN

It has long been evident that standard hemodynamic measurements provide an incomplete description of the contractile properties of the left ventricle, and do not permit the quantitative comparison of myocardial contractile state among different patients. Recent observations on the isolated papillary muscle and in intact canine left ventricles indicate that a comprehensive description of myocardial contractile performance requires consideration of the relations between myocardial wall tension, fiber length, and the velocity of fiber shortening. Accordingly, the mechanics of left ventricular (LV) contraction in man have been analyzed by correlating LV dimensional changes during contraction, determined directly from high speed cineangiograms, with simultaneous high fidelity recordings of LV pressure. The mechanical characteristics of ventricular contraction were expressed in a quantitative manner by deriving the extent and velocity of circumferential fiber shortening and LV wall tension (stress) throughout contraction. Patients with left ventricular disease were found to exhibit characteristic alterations in the time course of left ventricular wall tension as well as consistent reductions in the extent and maximum velocity of myocardial fiber shortening. In addition, left ventricular contractile state, estimated from the fiber shortening rate and the maximum wall tension, was consistently reduced in patients with left ventricular disease. These observations indicate that quantitative estimates of left performance in man can be derived from measurements of ventricular mechanics.

The mechanics of left ventricular contraction were then studied in ten patients with free aortic regurgitation by deriving myocardial wall tension and fiber characteristics throughout contraction from cineangiographic measurements of ventricular dimensions and high fidelity recordings of ventricular pressure. These studies demonstrated that left ventricular contractility, as estimated from the relation between fiber shortening rate and the maximum wall tension, was consistently reduced in four patients with hemodynamic evidence of LV failure. Further, characteristic alterations in the tension-velocity relation throughout contraction were observed in these patients when compared with that in patients without depression of myocardial function. In addition, in four of six patients without hemodynamic evidence of LV failure, mild depression of LV contractility was evidenced by reduced maximum fiber shortening rate and tension-velocity relations. In contrast, in all eight patients with severe mitral regurgitation studied to date, left ventricular force-velocity relations have been normal.

The maximum rate of intraventricular pressure development (peak dp/dt) has been used extensively in assessing myocardial contractility. However, this variable is also affected by alterations in ventricular afterload through opening of the semilunar valves. It was considered that the relationship between dp/dt and developed pressure $\frac{(dp/dt)}{P}$ throughout isovolumic contraction might afford a more accurate measure of contractility. In cat papillary muscles, intact dog heart, and in conscious man, this ratio was augmented by positive inotropic interventions but was unaltered by changes in loading. The determination of $\frac{(dp/dt)}{P}$ throughout isovolumic contraction appears to be a useful, simple, and experimentally valid approach to the assessment of the contractile state of the heart in intact man.

SECTION V

PHARMACOLOGIC INVESTIGATIONS

A. Autonomic Pharmacology:

An investigation was carried out to define the role of the beta adrenergic receptors in the regulation of peripheral vascular resistance and to determine whether norepinephrine released from nerve terminals acts at the same receptor sites in the arterial bed as injected norepinephrine. The hindlimb of the dog perfused at a constant flow rate was used as the test system. It was found that norepinephrine released from nerve endings in the arterial tree does not produce physiologically significant beta-receptor stimulation; humorally transported norepinephrine, however, stimulates both alpha and beta adrenergic receptors.

It has been shown that the extrinsically denervated feline heart manifests a "supersensitivity" to exogenous norepinephrine. Although the general phenomenon of denervation supersensitivity has been observed in virtually all systems of the body, the precise mechanisms underlying denervation supersensitivity of the heart have not been established. In order to study the role that changes in the postjunctional receptor site, as well as the loss of binding sites for catecholamines might play, the responses of the denervated heart to norepinephrine were compared with the responses of the same hearts to isoproterenol and calcium. The peak tension developed in the isovolumically beating left ventricle of isolated perfused hearts was used as an index of contractility. It was observed that while being supersensitive to norepinephrine, the denervated hearts showed a decrease in sensitivity at higher doses to isoproterenol. No changes were noted with calcium. These data support the view that the norepinephrine supersensitivity can be explained by a loss of intraneuronal binding loci for catecholamines.

The responses of normal and catecholamine depleted feline hearts to acetylcholine were compared using an isolated perfusion technique. The results demonstrated that acetylcholine has both negative and positive effects on the intact ventricle. The negative effects appear to be mediated by muscarinic receptors which are located on the muscle cells. The positive effects appear to be catecholamine dependent and mediated by nicotinic receptors.

In addition to producing a catecholamine mediated positive inotropic effect on ventricular myocardium, acetylcholine also antagonizes other catecholamine mediated effects on the heart. These apparently incongruous effects were studied in normal and catecholamine depleted, isolated, feline hearts using a perfusion technique. The results confirmed a norepinephrine-acetylcholine antagonism which appeared to be due to the muscarinic effects of acetylcholine.

The mechanism whereby aliphatic amines produce their sympathomimetic effects on the cardiovascular system has not been clear. Cyclohexylamine, a six-carbon cyclic aliphatic amine and hexylamine, a six-carbon straight-

chain aliphatic amine exert positive inotropic, chronotropic and pressor actions. These actions were studied in the intact dog and found to be significantly reduced after treatment with cocaine; these actions of cyclohexylamine were found to be abolished in animals pretreated with reserpine. Thus, hexylamine and cyclohexylamine exert their sympathomimetic effects by causing the release of neuronally-stored norepinephrine.

Catecholamines may exert their positive inotropic and chronotropic actions by activating the enzyme adenyl cyclase, which catalyzes the conversion of ATP to adenosine 3', 5'-monophosphate (cyclic AMP). Furthermore, glycagon is known to increase formation of cyclic AMP in many isolated tissues. The relevance of these observations to the heart was studied in isolated cat papillary muscle preparations, in spontaneously beating cat atria, in intact dog hearts and in isolated perfused dog hindlimbs. It was found that glucagon exerts powerful, positive inotropic and chronotropic effects. It produced small but significant decreases in peripheral vascular resistance. Single i.v. injections resulted in effects lasting 15 to 20 minutes. Propranolol did not prevent the inotropic responses but markedly decreased the chronotropic effects.

With this experimental background, a study was undertaken to evalute the cardiovascular effects of glucagon administration in man during the course of routine diagnostic catheterization. Administration of 3 to 5 mg. i.v. resulted in significant increases in cardiac index, mean arterial pressure, heart rate, and the maximum rate of left ventricular pressure development, with no significant change in left ventricular end-diastolic pressure or systemic vascular resistance. From these observations, it appears that glucagon may be a useful cardiotonic drug for the therapy of acute heart failure.

B. Digitalis Glycosides

Although paired electrical ventricular pacing overcomes digitalis induced arrhythmias in experimental animals, this procedure may be hazardous clinically. It was considered that ventricular pacing with a single stimulus might provide the same protective effect against arrhythmias produced by the glycoside. It was observed in the intact anesthetized dog that neither pacing nor potassium infusion altered the maximum tolerated dose of ouabain; however, arrhythmias were more effectively suppressed during ventricular pacing than during potassium administration. Thus ventricular pacing with a single electrical stimulus will effectively overcome serious digitalis induced arrhythmias, an observation which may have important clinical implications.

Although catecholamines and cardiac glycosides are both used to treat patients with heart failure, the relative inotropic effects of these two classes of drugs have not been defined. In order to study this problem, the relative inotropic effects of a catecholamine and a cardiac glycoside on the intact heart were evaluated by infusing increasing concentrations of isoproterenol and ouabain into open-chest anesthetized dogs to the point of toxicity. Cardiac output and myocardial contractile force measured with a strain gauge arch on the right ventricle and by the first derivative of

17

the left ventricular pressure pulse were considerably greater with isoproterenol than with ouabain just prior to the toxic doses. Following suppression of toxic arrhythmias by ventricular pacing, additional infusion of ouabain increased contractile force to the same peak level achieved with isoproterenol but without further augmentation of cardiac output. Moreover, the peak effect achieved by the catecholamine appeared to be a ceiling, since it was not exceeded by the prior administration of large doses of the glycoside.

C. Other Pharmacologic Agents

Despite considerable interest in the cardiocirculatory effects of ethanol (EtOH), the direct actions on ventricular myocardium have not been well understood. Effects of the clinically meaningful blood concentration range of 100 to 500 mg.% EtOH were studied on both isotonic and isometric contractions of isolated right ventricular cat papillary muscles suspended in oxygenated Krebs solution in a myograph and electrically stimulated at a frequency of 12/min. Depressions of the velocity of isotonic contraction, the rate of isometric tension development and the total isometric force developed in normal cat papillary muscle and in papillary muscles from cats with chronic right heart failure due to pulmonary artery constriction were observed. It is concluded that ethanol exerts a direct negative inotropic effect on heart muscle.

Although angiographic contrast media produce marked changes in circulatory dynamics, their effects on the contractile state of the myocardium have not been completely defined. Accordingly, the influence of Hypaque was studied on the contractile properties of cat papillary muscles. Angiographic dye, even in small concentrations (10% Hypaque) was found to exert a profound direct negative inotropic action on heart muscle which appears to be largely responsible for the transient hypotension observed clinically.

Although intravascular infusion of hyperosmotic solutions has become increasingly common in clinical diagnostic and therapeutic programs in recent years, the effects of increased osmolality on myocardial mechanical characteristics have not been fully defined. Accordingly, it appeared desirable to compare the mechanical characteristics of in vitro cat right ventricular papillary muscle preparations in iso-osmolal bathing solutions (normal Krebs) and in hyperosmolal bathing solutions. It was found that relatively low levels of hyperosmolality (< 100 mOsm/Kg H_2O above control) are associated with an enhancement of contractile force, and that high levels (<200 mOsm above control) are associated with a depression of contractile force and an increase in the stiffness of the series elastic component.

Although nephro-toxicity, neurotoxicity and ototoxicity are frequent concomitants of antibiotic therapy, direct cardiac toxicity has not been recognized. Interest in this possible toxic effect grew out of the observation that persistent hypotension was a feature of the clinical course of a patient who manifested signs of streptomycin toxicity. Dose-response curves, measuring left ventricular dp/dt and right ventricular wall

tension and utilizing between 2.5 mg/Kg and 40 mg/Kg streptomycin intravenously were obtained and revealed a 9 to 37% decline in LV dp/dt and a 12 to 42% decline in the right ventricular wall tension. In the isolated perfused Langendorf cat heart preparation, depression in isovolumetric pressure generation was observed when streptomycin was added to the perfusate.

Similar depressions in left ventricular function and in the isolated Langendorf preparation were seen when vancomycin, tetracycline, kanamycin and colymycin were tested. All of the agents were administered in concentrations commonly achieved clinically.

SECTION VI

STUDIES ON PATIENTS WITH CONGENITAL AND ACQUIRED HEART DISEASE

A. Diagnostic Techniques

The recent development of the instantaneously sensing gamma-scintillation camera and the rapidly scanning television tube, capable of recording wide field images over the precordium produced by trace concentrations of gamma emitting radioisotopes at 1/60 sec. intervals as it flows through the circulation, and the storage of the radioisotope picture on video magnetic tape, has provided a means for visualizing the anatomic features of the heart and great vessels without the hazards involved in introducing a radiopaque agent. Sodium pertechnetate ^{99}m was rapidly injected into selected cardiac chambers at the time of diagnostic catheterization in 50 patients with a variety of congenital and acquired forms of heart disease. Movement of radioisotope closely reflected the hemodynamic alterations caused by these conditions. The radioisotope-angiocardiogram was found to provide a new approach for the visualization of the cardiovascular system, does not require the use of radiopaque media, is safer and does not disturb circulatory function.

The use of image intensification and closed circuit television during fluoroscopy has allowed the development of a more precise method for recording the movements of cardiovascular structures. Such a technique, heart motion video tracking (Radarkymography), was developed and through its use motion of any cardiac border can be translated into a reproducible linear graphic tracing. More than 100 patients with a variety of congenital and rheumatic heart lesions were studied. Characteristic graphic linear tracings were obtained in patients with mitral valve disease, aortic outflow tract obstruction, ventricular aneurysm and coarctation of the aorta. The radarkymogram was found to offer certain advantages over the electrokymogram, which to date has been the major technique for recording motion of the cardiac silhouette.

B. Studies on Idiopathic Hypertrophic Subaortic Stenosis

The clinical, hemodynamic and angiographic findings were determined in six patients with intraventricular pressure differences resulting from catheter entrapment within portions of the left ventricle obliterated during systole. These findings were contrasted with observations in 19 patients with idiopathic hypertrophic subaortic stenosis (IHSS) with documented obstruction to left ventricular outflow. Cardiac symptoms, including angina and syncope, occurred in both groups of patients. While a cardiac murmur was noted in each patient, the murmur in those with non-obstructive pressure differences was soft and non-specific and was not accompanied by a thrill or by paradoxical splitting of the second heart sound with respiration. The intraventricular pressure difference was augmented to comparable levels in patients with obstruction and those with non-obstructive pressure differences; however, in the latter group, the bifid arterial pulse contour and abnormal arterial pulse pressure response following a ventricular extrasystole, typical of IHSS with obstruction, were not observed. While angiographic

evidence of obstruction was not observed in patients with non-obstructive pressure differences, four of these six patients exhibited asymmetric ventricular hypertrophy involving the interventricular septum. It is postulated, on the basis of these observations, that asymmetric ventricular hypertrophy may be responsible for either obstruction to left ventricular outflow or apical obliteration and non-obstructive pressure differences, but that distinctive differences in the clinical and laboratory manifestations of asymmetric hypertrophy occur as the result of obstruction.

Considerable controversy exists concerning the angiographic anatomy of the left ventricle (LV) during systole in patients with IHSS and intraventricular pressure gradients. LV angiocardiograms were reviewed in detail in 58 patients with IHSS. In 83% of the technically satisfactory studies, in the frontal projection a linear radiolucent area extended across the LV outflow tract during systole at a level corresponding to the site of the intraventricular pressure change; the anterior mitral leaflet was identified in diastole, and during systole this leaflet did not retract normally but projected anteriorly. In addition, the axis of the papillary muscles was abnormal. Thus, the anterior leaflet is held in the outflow tract, where it meets the hypertrophied interventricular septum in midsystole, and it is proposed that this mechanism plays an important role in producing mechanical obstruction to ejection in many patients with IHSS.

Since the obstruction in IHSS is dynamic, whereas that in discrete aortic stenosis (AS) is fixed, it was considered that the shape of the transaortic pressure gradient might be altered in IHSS and thereby provide a means of differentiating these two conditions. Early arterial and late ventricular pressure peaks were observed in IHSS and are suggested to be related to absence of obstruction to ejection early in systole. Further, the ratio of the mean pressure gradient during the first half of ejection to that of the last half was less than 1.0 in IHSS, and greater than 1.0 in AS. Thus, analysis of the configuration of the transaortic pressure gradient affords a reliable separation of AS from IHSS, is of even greater diagnostic value than previous descriptions of hemodynamic events, and does not require a provocative maneuver.

The efficacy of beta adrenergic receptor blockade in relieving angina pectoris was demonstrated in four of seven patients with IHSS originally studied 24-30 months ago. Treadmill exercise testing was utilized. Two of the four patients who initially showed marked improvement slowly became symptomatic on 160 mg. propranolol daily and each required operation at 18 months and 19 months respectively after initiation of therapy. Repeat cardiac catheterization in these two patients demonstrated an increase in resting left ventricular-aortic gradient in one and a decrease in the other when compared with the initial study prior to chronic propranolol therapy. Two patients have maintained improvement for 18 and 29 months respectively but repeat exercise testing in one of them revealed a 30% reduction in exercise tolerance. Of the three patients who initially showed little or no improvement on propranolol therapy, two have been operated upon with improvement of angina pectoris.

Several recent reports present conflicting evidence concerning improvement in patients with IHSS after administration of beta adrenergic blocking drugs. Our findings suggest that in some patients with angina pectoris due

to IHSS, propranolol may cause initial amelioration of symptoms, but may not obviate the ultimate requirement for operation.

C. Studies on Patients with Coronary Artery Disease

Carotid sinus massage can relieve angina pectoris and has been utilized as a diagnostic test for angina. Stimulation of the carotid sinuses abolishes angina by lowering the three most important determinants of the energy needs of the heart; i.e., ventricular pressure, heart rate and myocardial contractility. Accordingly, it was considered that electrical stimulation of the carotid sinus nerves might be of clinical value in the treatment of angina pectoris. In 10 men and 3 women, most with well documented previous myocardial infarctions and all with severe, incapacitating angina pectoris, bipolar platinum electrodes were attached to the carotid sinus nerves. The latter were connected to a subcutaneously placed radio-frequency receiving unit that the patients could activate on demand by a radio-frequency transmitter.

In seven patients studied postoperatively stimulation of the nerves lowered arterial pressure, heart rate and the pressure-rate product, a hemodynamic index related to myocardial oxygen needs, both at rest and during exertion. Anginal episodes could be abolished immediately in each of these patients by activating the stimulator, and prophylactic activation allowed them to engage in activities that otherwise were consistently associated with severe pain. Also, stimulation allowed a marked prolongation of the duration with which each patient could pedal a bicycle ergometer and increased significantly the level of work that could be tolerated before angina occurred.

Of the remaining patients, 3 are still recovering from the operation and stimulation has not as yet been attempted; one patient reacts to stimulation with only minimal decreases in heart rate and arterial pressure, and two patients died shortly after operation from acute myocardial infarction.

Preliminary hemodynamic studies in 4 patients have shown that stimulation both at rest and during supine exercise causes a marked decrease in forearm vascular resistance, total peripheral resistance and arterial pressure, and smaller reductions in cardiac output and heart rate. In addition, stimulation of the carotid sinus nerves does not appear to alter venous tone as measured by the occluded limb technique, nor does it diminish the increase in venous tone that occurs with exercise. The operation is relatively simple, the instrumentation is commercially available, and the early results are encouraging enough to warrant cautious trial in a larger number of patients with coronary artery disease and severe, incapacitating angina pectoris.

An effort was made to define the clinical spectrum of mitral regurgitation in patients with papillary muscle rupture or dysfunction due to myocardial infarction. Rupture of a papillary muscle due to myocardial infarction is generally considered to be a fatal lesion while papillary muscle dysfunction is generally well tolerated. However, four patients with mitral regurgitation due to papillary muscle rupture have been operated upon and all

have derived significant benefit from mitral valve replacement. The patients ranged from 51 to 69 years of age and were operated upon at intervals from 3 to 15 months following myocardial infarction. Each patient was in congestive heart failure and was in sinus rhythm; three of the four had severe pulmonary hypertension (60 to 80 mm. Hg) and grossly elevated mean left atrial pressure with "v" waves 37 to 49 mm Hg. A fifth patient whose clinical and hemodynamic picture was indistinguishable from that of the patients with papillary muscle rupture was found at operation to have intact but fibrotic papillary muscles. This experience reflects the clinical spectrum possible when mitral regurgitation follows myocardial infarction. Operative intervention should be considered when the clinical course of the patient shows continuing deterioration despite medical therapy.

Clinical observers have long recognized that angina pectoris occurs more readily when exertion is performed in a cold rather than in a warm environment. To elucidate the physiologic basis for this observation, 6 patients with angina due to coronary artery disease (CAD) and 5 subjects without CAD with either mild or no impairment of cardiac function were studied at rest and during the same level of mild upright exercise at 2 ambient temperatures: 23°C and 15°C. The effects of cold were similar in both groups of subjects. It was observed that a cold environment provokes an increase in total peripheral resistance at rest and during exercise. The consequent rise in arterial pressure, by augmenting myocardial O_2 requirements, would thus contribute to an earlier onset on angina.

SECTION VII

STUDIES ON THE PERIPHERAL CIRCULATION

In the past it has been assumed that arterioles and veins in skin and muscle react similarly to a variety of stimuli and that veins and arterioles respond in like manner. These hypotheses have not, however, been tested critically.

In order to evaluate the relative participation of the skin and muscle vascular beds in the reflex alteration of peripheral vascular resistance, arterial pressure and forearm blood flow were measured in 6 normal subjects in which the skin circulation in one forearm was temporarily suppressed by epinephrine iontophoresis. When baroreceptor activity was inhibited by application of lower body negative pressure, calculated vascular resistances were significantly higher for both skin and muscle vessels. Conversely, enhancement of baroreceptor activity by negative pressure around the neck produced significant decreases in both skin and muscle vascular resistance. Thus, it appears that both skin and muscle resistance vessels participate in the reflex regulation of arterial pressure through the baroreceptor mechanism.

To determine the relative participation of skin and muscle capacitance beds of the forearm in venomotor reflexes, epinephrine iontophoresis was combined with forearm plethysmography so that the volume of muscle veins could be estimated simultaneously with the volume of cutaneous veins, at a constant venous pressure. With this technique not only are the cutaneous veins markedly constricted but they also are prevented from filling since skin blood flow is abolished. A variety of veno-constrictor stimuli were employed, including leg exercise, deep breathing and the application of ice to the forehead. It was observed that only the cutaneous veins participate in venomotor reflexes in the forearm. Further, since the forearm is principally composed of skeletal muscle and the hand skin, an explanation is provided for the observation that veins of the forearm, studied as a whole, appear less reactive to stimuli than veins of the hand.

Although in systemic amyloidosis there is extensive pathologic involvement of the blood vessels of many organs, little is known concerning the functional significance of this abnormality in the peripheral vascular system. Accordingly, the response of the resistance bed in the forearm to the restoration of circulation after inflow occlusion and to vigorous forearm exercise were compared in 8 patients with primary amyloidosis and in 20 normal subjects. The results suggest that the muscle pain often experienced by patients with systemic amyloidosis is, at least in part, the result of claudication secondary to diseased arterioles leading to a diminished arteriolar dilator capacity and compromised blood supply during exertion.

SECTION VIII

OTHER PHYSIOLOGIC INVESTIGATIONS

In earlier studies it was shown that the anterior leaflet of the mitral valve contains blood vessels, nerve fibers, and cardiac muscle in addition to elastic fibers and collagen. When studied in a myograph, it actively developed tension and shortened. This tension was increased by norepinephrine and decreased by acetylcholine. In situ surface electrocardiograms have been recorded corresponding in time to ventricular activation. It was felt, therefore, that by establishing the wave form of the intracellular action potential in the mitral valve muscle cells, it could be determined whether their activation was atrial or ventricular. The recordings appear to possess a typical ventricular action potential wave form, suggesting that this muscle is fundamentally ventricular in nature.

In experimental heart failure, myofibrillar ATPase activity has been found to be depressed. This depression has been correlated with reduced contractility of the associated right ventricular papillary muscles. It has been proposed that myofibrillar ATPase activity may be directly related to alterations in the contractile state of the heart. In order to test this hypothesis, drugs known to increase the contractile state of the heart were added to myofibrillar preparations. In addition to these in vitro interventions, contractility was altered in vivo by inducing hyperthyroidism. ATPase activity was then assessed in myofibrillar preparations made from the hearts of these animals. These results suggest that myofibrillar ATPase activity is not directly related to pharmacologic alterations of the contractile state of the heart.

Although prolongation of the contractile process of skeletal muscle in myxedema has been documented in man, the effects of hyper- and hypothyroidism on the intrinsic function of isolated skeletal muscle are not clear. Accordingly, contractile properties of soleus muscles of euthyroid, hyperthyroid and hypothyroid rats were studied in a myograph. Tetanic contractions at a stimulation frequency of 100/sec were examined.

Maximum isometric tension was found to be essentially identical in muscles from hyperthyroid and euthyroid animals, but was significantly depressed in muscles from hypothyroid rats. The rate of tension development was increased in hyperthyroid and decreased in the hypothyroid muscles. The duration of active state was also altered by changes in thyroid function; it was shortened in hyperthyroidism and prolonged in hypothyroidism. The mean rate of relaxation was increased above the normal value in hyperthyroid muscles and slowed in the hypothyroid muscles. Thus, it is apparent that hyperthyroidism and myxedema result in profound alterations in the intrinsic contractile properties of skeletal muscle.

An investigation was carried out in order to determine whether activation of pulmonary stretch receptors causes reflex changes in the cardiovascular system. The lungs of dogs were distended to various positive pressures, and the changes produced in the cardiovascular system were assessed by measuring peripheral vascular resistance, heart rate, and the force of myocardial contraction. When the lungs were suddenly distended, marked vasodilation occurred in every instance, heart rate generally slowed,

and myocardial contractile force decreased. After vagotomy, the cardiovascular response to sudden lung inflation was either abolished or markedly attenuated. Thus, this reflex links the pulmonary to the cardiovascular system.

It has been observed at cardiac catheterization that many newborn infants have a pressure gradient between the main and peripheral pulmonary arteries. These gradients, at times are large enough to cause significant differences in the pulmonary resistance when calculated by using main and peripheral mean pulmonary artery pressures. On recatheterization of several of these children at an older age, this gradient was no longer present. Since most of the children in whom these observations were made had some type of congenital cardiac defect, it was of interest to determine whether this gradient exists in otherwise normal hearts and to measure the pressures without a catheter obstructing the vessel in which the pressure measurement was being made.

A pressure gradient was found at the bifurcation of the main pulmonary artery in the newborn lamb and it was observed that this gradient disappears at about 2 - 3 weeks of age but can be evoked in the older animal up to about 2 months of age by interventions which increase pulmonary blood flow. This gradient provides an additional reason for the elevated pulmonary vascular resistance in the newborn animal. Consequently, the peripheral pulmonary artery pressure should be used for evaluation of pulmonary resistance at the muscular artery and arteriolar levels in newborn infants, especially if a defect resulting in elevated levels of pulmonary blood flow is present.

Although it is generally appreciated that the hemodynamic effects of exercise are influenced by changes in body position and the form of exercise performed, very little is known about the significance of these factors in patients with impaired cardiac function. Accordingly, a comparison of the circulatory response to supine bicycle and upright treadmill exercise was undertaken in 8 patients with hemodynamically insigificant cardiac defects and in 9 patients with moderate to severe cardiac impairment. In the former group cardiac output (C.O.), stroke volume (S.V.), and pulmonary arterial pressure (P.A.P.) were slightly but consistently lower at any given O_2 consumption in the upright position as compared to supine. In the patients, however, C. O. and S.V. did not differ significantly during comparable levels of exercise in the two positions but mean P.A.P. were considerably lower, by an average of 17 mm Hg during upright exercise. In an additional group of 8 patients heart rates and blood pressures were higher during both supine and upright exercise on the bicycle than during treadmill exercise. Thus, both changes in body position and the type of exercise performed significantly influenced the hemodynamic response to exercise.

ANNUAL REPORT OF THE SECTION ON CLINICAL BIOPHYSICS, CARDIOLOGY BRANCH, NATIONAL HEART INSTITUTE - July 1, 1967 through June 30, 1968

The program of this section has as its broad objectives the application of biophysical disciplines to the study of experimental pathology. Of particular interest are studies which bear on the mechanisms of pulmonary and vascular diseases. Thus, it is convenient to discuss this program under two headings, cardiovascular, and pulmonary.

Cardiovascular:

Historically pathologists have suspected that the rather consistent topology of atherosclerotic lesions may be related in some manner to associated hydromechanical forces. Several projects have been designed to explore these ideas and to establish mechanical and chemical parameters which may be used, at least tentatively, to define the mechanical "strength" and apparent chemical "change" of the endothelial surface and vascular wall. One project was completed and published this year in which it was shown that the endothelial surface will rapidly degenerate in regions where velocity gradients in the adjacent blood flow become sufficiently large. If the shearing stress associated with these velocity gradients exceeds 300 to 400 dynes/cm^2 for periods as short as one hour, the endothelial surface can be seen to deteriorate rapidly in the following sequence: when the shearing stress exceeds a critical value (τ_c), the endothelial cell is transformed from an essentially elastic body to an apparent viscous body which deforms progressively with time ultimately becoming unstable and washing away with the stream. This deformation is associated with apparent alterations in the physicochemical properties of the cell as evidenced by cellular swelling, granulation, and severely altered staining properties.

These observations have been pursued by a new series of studies which have been designed to study the relationships among the apparently altered physicochemical properties of the intimal surface, associated exposure to shearing stress, and changes in the cytology of the endothelial cell populations.

By using a specially designed nontraumatic intra-aortic device to produce a wide range of hydraulic shearing stress along a segment of otherwise unmolested endothelial surface, a variety of associated cytologic and physicochemical changes were produced. The details of this technique have been presented previously. In the present group of studies Evans blue dye was given the animals to form a visual tag for albumin so that areas of endothelial surface which become more permeable to albumin, and presumably other serum proteins, will stain blue. In addition, an artificial fat emulsion (Intralipid) was given parenterally to study its affinity for the normal and injured intimal surface. Photographic and densimetric techniques were developed to estimate the intimal concentration of Evans blue dye. Histologic techniques were developed to estimate the population densities of normal and abnormal endothelial cells, as well as the percent surface area involved with increased fat and fibrin deposition. These studies have confirmed and extended the observations of the previous study. The acute critical stress

for endothelial cells in this group of animals did not vary significantly from the previous group. In addition to this parameter, which defines the elastic limit of the endothelial surface, a second parameter was defined called the "yield stress" (τ_y). The yield stress of the endothelial cell population defines the stress in a given study at which the viscous cells reach their critical deformation and leave the basement membrane. The product of this yield stress times the duration of the study represents the "viscous resistance" of the cells to deformation and thus may be thought of as a parameter defining the "dynamic" rigidity of the endothelial cells. For stress exposure in excess of the yield stress, the endothelial cells are totally eroded from the basement membrane, subjecting it, in turn, to the forces of the adjacent flow.

Strong correlations were found between the duration of exposure of the basement membrane and the depositions of both fat and fibrin. In addition, the concentration of Evans blue dye in the endothelial surface was found to have a high correlation with stress exposure, both in the area of normal cell population as well as in the area of abnormal cell population and erosion.

These studies clearly demonstrate that acute endothelial damage, even to the point of total erosion, can occur from purely hydraulic forces or shearing stresses which are only slightly higher than those which can occur under extreme physiologic circumstances. Measureable parameters of the endothelial strength to resist these forces have been defined which will be tools in further study of experimental pathology. Similarly, measureable physicochemical changes which are the analogs of certain vascular processes also have been defined. A series of studies have been designed and are in progress to relate the foregoing observation progressively more closely to their disease counterparts.

In an effort to gain deeper insight into the aforementioned physicochemical changes, studies are in progress to develop techniques to define these changes more rigorously in chemical terms. As a first step towards studying the electrochemical potential at the blood vessel wall interface it is necessary to establish techniques for measuring electrical potential in this complicated milieu. An assortment of different types of electrodes have been studied to determine their possible usefulness in the measurement of the electrical potential of the endothelial surface. A Bak recording electrometer, having an input impedance of 10^{9} ohms, was used to sense the electrical potential from each of the electrodes. Each of the following types of electrodes were studied: metallic silver-silver chloride, bright platinum, silver-silver chloride-potassium chloride micropipette electrodes, and silver-silver chloride-sodium chloride micropipette electrodes. Paired electrodes were used for the active and indifferent electrodes in the system. Excised fresh aortic tissue in either oxygenated heparinized blood or in oxygenated Krebs solutions were studied. The indifferent electrode was placed in the solution at some point not in contact with the tissue and the active electrode was placed in a recording micrometer so that it could be advanced slowly toward the endothelial surface. Prior to contact with the endothelial surface the recorded potential varied around zero $\pm$ 5 millivolts. At the instant of contact, in each case, the potential instantly dropped to a

negative 10 to 20 millivolts depending on the degree of advancement into the tissue. The subsequent time course of voltage depended on the particular electrode. The voltage from the micropipettes usually remained fairly stable at a negative value. That from the larger diameter metallic electrodes demonstrated a progressive increase in the positive direction approaching baseline values.

Information about the interfacial electrochemical potential of the endothelial surface and its relationship to the associated hydrodynamic events is of considerable importance, both to our knowledge of vascular thrombosis and to furthering our understanding of the pathogenesis of certain arterial disease processes. In particular the electrical potential is of considerable interest since the cellular elements (e.g. platelets), and the macromolecules in the blood (e.g. lipoproteins) appear to be slightly negatively charged at the normal physiologic pH of the blood. It is the interaction of these charges with the field created by the charged endothelial surface which will determine whether there is a net electrical force driving these elements toward or away from the wall. Any unique interpretation of the measured potentials is hazardous in light of the present scanty knowledge about the electrochemistry of electrodes passing from one chemical milieu into another, such is the case in the present preparation. Future efforts will be directed toward seeking collaborative assistance from consultants in surface chemistry, as well as from the Departments of Physics and Chemistry at American University, both of whom have expressed interest and had experience in allied areas.

The foregoing studies have been directed toward a study of the intimal rheology and the "strength" of the intimal surface to resist applied stresses. It is equally important to establish the levels of stress to which the normal endothelial surface is exposed. Modern hemodynamic theories do not permit calculation of these stresses from simple pressure and flow measurements. Therefore, it is necessary to develop techniques for estimating directly the velocity fields in the living arterial system and the associated velocity gradients in the vicinity of the intimal surface. Projects were undertaken to develop methods to measure these blood velocity fields, turbulence, and the associated shearing stresses on the vessel wall at various locations along the system in the living animal. A constant-temperature, heated-film anemometer system was developed in conjunction with the Department of Applied Physics and Space Sciences at Catholic University for these in vivo measurements. Two types of sensing probes were developed: a velocity probe, and a velocity gradient or fluid shear stress probe. The sensing element in each consists of a thin platinum film fused to the tip of a 20 gauge needle. As the fluid passes this tip, heat is convected away at a rate which is a function of the velocity or the velocity gradient depending on the mode of operation and configuration of these probes. The probes were evaluated for steady and pulsatile flow in rigid circular tubes using both glycerin-water mixtures and blood. In vivo studies were done making measurements along the thoracic aorta of anesthetized dogs and pigs. These devices were studied in physical models of blood vessels in which the flow profiles could be predicted by well-established theory. Observations were found to agree very well with the true or predicted velocity fields. Moreover, the integrated velocity profiles that were measured correlated well with the simultaneously recorded flow

values using orifice meter and electromagnetic flowmeter techniques. In the animal studies the velocity measurements along the aorta indicated that the velocity profiles were considerably more blunt than would be predicted by the currently held linearized theories of hemodynamics. The integrated flow pulse forms obtained by the heated-film technique were similar in magnitude and contour to those obtained simultaneously from an electromagnetic flowmeter. Fully developed turbulent flow was not observed; however, occasional eddy turbulence was found to occur in the aortic arch of dogs weighing less than 30 kg. Preliminary measurements of lumen surface shear stress by the shear probe technique have shown that the peak wall shear stresses are approximately one-third of the critical stress described above. This method should provide a powerful tool in quantitative investigations of the vascular system. These techniques will be extended to study various vascular configurations along the aorta and its major branches. Particular emphasis will be given to critical areas, such as branch sites in the aorta, coronary arteries, and renal arteries.

Preliminary results from these studies are of considerable interest in that it appears that the average shearing stress on the wall of smaller arteries, in particular the coronary artery appears to be about 25% of the critical stress measured in the above studies. This suggests that there is not a wide margin between the stresses to which a vessel surface is normally exposed and the strength of that surface.

The studies described above have dealt primarily with the rheology of the intimal blood interface. The rheology of the rest of the arterial wall has been the object of study in this laboratory for a number of years. By using techniques and methodology that have been described previously these studies have been extended to establish the elastic moduli of the vessel wall in the three principle directions (the circumferential, longitudinal, and radial). In all such calculations there are two assumptions; namely, that the vessel wall is incompressible and that an "elastic symmetry" exists in the arterial segment such that one need be concerned only with the principle directions of strain and stress. Two studies were done to test these two critical assumptions.

The compressibility of the blood-vessel wall was examined using a specially designed hydraulic chamber. The blood vessel wall was found to be essentially incompressible. The value of the bulk modulus was 4.4×10^6 gms/cm^2 and the maximum volume strain was 0.06%. The errors introduced in values calculated for the elastic moduli due to the incompressibility assumption were estimated to be less than 0.1%.

The elastic symmetry of blood vessels was studied by measuring the shearing strains produced under normal physiologic loading. The resulting shearing strains were always an order of magnitude smaller than the comparable values of longitudinal and circumferential strains. It can be concluded that blood vessels demonstrated remarkable elastic symmetry, a fact which greatly simplifies the analysis of elastic properties in a cylindrical tube. These data are of considerable importance in advancing our knowledge of vascular rheology and are essential to any theoretical treatment of circulatory dynamics.

Our program in defining myocardial mechanics has advanced in two general areas: instrumentation and the histologic anatomy of the heart wall. An instrument was developed and evaluated for measuring the average tension developed across the myocardial wall in the living, beating heart. This force gauge was designed to couple into the heart wall with two sets of parallel gold-plated tines. The segment of tissue between the sets of tines is then compressed after insertion so that the intervening muscle fibers are shortened below their point of contraction on the length-tension diagram. These fibers, therefore, no longer counterbalance the tension developed in the neighboring tissue which has now become supported by the tines of the force gauge. This force is sensed electrically and amplified. The details of design and response characteristics have been published.

Detailed histologic studies of the myocardial wall from hearts stopped in systole and in diastole were carried out to measure the distribution and orientation of muscle fibers in the wall in these two states. It was found that the left ventricle may be described as a laminated structure in which the fibers are remarkably parallel in each lamina but have a continuous change in angular orientation across the wall. These data are essential in developing any realistic mathematical model of the myocardial wall and in establishing the relationship between the laws of muscle fiber contraction and the overall system mechanics.

Pulmonary Studies:

Activities in the pulmonary area have revolved around development of mathematical descriptions of flow through collapsible tubes and the development of experimental techniques to study the parameters of pulmonary mechanics in small animals, in particular, the rabbit. The rabbit appears to be a particularly useful animal for study of experimental pathology of pulmonary obstructive disease such as emphysema. Apart from the obvious economic advantages of studying rabbit colonies as opposed to larger animals, the rabbit has two particular advantages. In the first place rabbits older than two years have been shown to develop spontaneous pulmonary lesions which appear to be histologically identical to the lesions seen pathologically in man with pulmonary obstructive disease. Secondly, young rabbits develop massive chondrolysis within 24 hours following intravenous infusion of papain. Our studies have shown that the cartilage of the bronchial tree is involved in this chondrolysis.

The instrumentation and methodology described previously were used to study groups of rabbits in various age groups as well as rabbits before and after the administration of papain. Normal parameters of ventilatory function, including lung compartments, compliance values, airway resistance values, and effective pulmonary time constants, were established for the various age groups in the normal control series. Apart from differences in the size of the lung compartments no significant differences were found for the parameters among the various age groups when data were normalized according to lung size. However, in animals studied before and after receiving intravenous papain, marked changes in the pulmonary expiratory flow-volume relationship was found. The marked depression in maximum respiratory flow was

attributed to the increased collapsibility of the bronchial tree. Lung compliance was not significantly altered by intravenous papain. These studies are being prepared for publication and will not be pursued further at NIH.

Efforts to improve the mathematical description of flow through collapsible tubes have continued with particular emphasis on improved computational techniques. A study of these mathematical models, particularly that of the bronchial tree, has been of importance in giving insight into the details of airway dynamics which would not otherwise be available since measurement techniques do not exist which will permit direct observation of the bronchial tree in vivo. For example, one cen only calculate the distribution of turbulence, wall-shearing stress, expulsive forces of the respiratory stream, stresses of deformation on the bronchial wall, and the effects of altered gas density or viscosity along the tree since these cannot be measured directly. A study of these mathematical models has suggested certain interesting possibilities concerned with the pathogenesis of pulmonary emphysema. It is of interest to note that the computed shearing stresses that can be developed along the bronchial tree may become extremely high during forced expiratory efforts. It is possible that the magnitudes of these stresses might exceed the "yield stress" of the bronchial epithelial cells resulting in erosion of this delicate surface. Moreover at these levels of stress considerable heat is generated which could result in thermal injury. These suggestions are being pursued along two different avenues, the first is to develop techniques of measuring the necessary bronchial parameters more accurately so that the model may be endowed with more realistic values than are currently available. The second avenue will be to study the errors introduced in the numerical procedures involved in solution of the foregoing systems of equations. These studies will be undertaken in conjunction with the mathematicians in the Computer Division.

In summary, the major efforts of this section have been directed towards solution of basic problems in rheology, fluid mechanics, and histology, and toward solution of the ancillary instrumentation problems to implement these studies. Significant progress has been made toward applying these disciplines to our ultimate target, vascular disease and pulmonary emphysema. These programs have required active consultation, both intramurally and extramurally with specialists in the basic chemical, computer, and physical sciences. A number of fruitful collaborative efforts have ensued and a number of new areas opened by these programs.

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Project Title: Comparison of the Peak Inotropic Effects of a Catecholamine and a Digitalis Glycoside in the Intact Canine Heart

Previous Serious Number: None

Principal Investigator: G. David Beiser, M.D.

Other Investigators: Stephen E. Epstein, M.D.
Morris Stampfer, M.D.
Robert E. Goldstein, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: Although catecholamines and cardiac glycosides may both be used to treat patients with heart failure, the relative inotropic effects of these two classes of drugs have not been defined. Increasing concentrations of isoproterenol and ouabain were infused separately into 7 open-chest anesthetized dogs until toxicity developed. Myocardial contractile force, measured with a strain gauge arch on the right ventricle, and cardiac output, measured with a flowmeter, were compared just prior to toxicity. Myocardial contractile force was greater with isoproterenol than with ouabain, averaging 35 vs. 22 mm ($p < .05$). Similar results were obtained when arterial pressure was held constant and when the heart was paced at the rate achieved during the peak isoproterenol effect. When the arrhythmias following toxic doses of ouabain were corrected with either KCl or diphenylhydantoin, infusion of isoproterenol still produced substantial augmentation of myocardial contractile force (20 to 33 mm, $p < .005$), LV dp/dt (2870 to 5700 mm Hg/sec, $p < .025$) and CO (1.2 to 1.6 L/min), to levels comparable to those obtained with maximal doses of isoproterenol alone.

In an additional group of 7 dogs when the ouabain induced arrhythmias were suppressed by ventricular pacing, the further infusion of ouabain resulted in an increase in myocardial contractile force to the same peak levels achieved with isoproterenol. However, despite this marked increase in contractility (22 to 35 mm, $p < .005$) and with arterial pressure maintained constant, cardiac output and stroke volume did not increase beyond

the values achieved at subtoxic doses of ouabain.

In conclusion, at subtoxic doses the maximal inotropic effect of isoproterenol exceeds that of ouabain in the intact heart. With suppression of arrhythmias by ventricular pacing, continued infusion of ouabain increases contractile force to the same peak level achieved with isoproterenol but does not further augment the performance of the heart as a pump. Moreover, the peak effect achieved by the catecholamine appears to be a ceiling, since it is not modified by prior administration of large doses of glycoside.

Proposed Course of Project: Project continuing. Additional inotropic agents will be evaluated and dogs with experimental heart failure will be studied.

Honors and Awards: None

Publications: None

Serial No.- NHI-33(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Participation of Both Skin and Muscle Resistance Vessels in Baroreceptor Reflexes

Previous Serial Number: None

Principal Investigator: G. David Beiser, M.D.

Other Investigators: Robert Zelis, M.D.
Stephen E. Epstein, M.D.
Dean T. Mason, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Others:	.4

Project Description: Although reflex alterations of peripheral vascular resistance in response to changes in baroreceptor activity provide one of the more important compensatory mechanisms for maintenance of arterial pressure, it is not clear whether the peripheral cutaneous, or the muscular vascular beds, or both, participate in this reflex. In normal subjects baroreceptor nerve activity was inhibited by application of lower body negative pressure (LBNP) and enhanced by application of neck negative pressure (NNP). Forearm blood flow was measured with a strain gauge plethysmograph simultaneously in both arms. In one arm the skin circulation was temporarily arrested by epinephrine iontophoresis. Arterial pressure was measured directly and total forearm (FVR), muscle (MVR) and skin (SVR) vascular resistances calculated before and during each intervention. During LBNP there was no significant change in mean BP; however, heart rate increased from 59 to 69 ($p < .005$) and FVR from 20 to 38 mm Hg/ml/min/100 gm, ($p < .001$). Both skin and muscle vessels contributed to the increase in FVR: SVR increased from 47 to 110 ($p < .05$), and MVR from 43 to 72 ($p < .005$). During NNP BP decreased from 89 to 75 mm Hg ($p < .005$), heart rate from 60 to 55 ($p < .005$), FVR from 36 to 24 ($p < .025$). Both skin and muscle vessels contributed to the decrease in FVR: SVR decreased from 75 to 49 ($p < .05$) and MVR from 68 to 51 ($p < .005$). These results indicate that both the skin and muscle resistance vessels participate in the reflex changes initiated by alterations in baroreceptor activity.
Proposed Course of Project: Project completed.
Honors and Awards: None
Publications: Manuscript in preparation.

145

Serial No. - NHI-34 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Comparative Hemodynamic Effects of Treadmill and Bicycle Exercise in Patients with Cardiac Impairment.

Previous Serial Number: NIH-28(c)

Principal Investigator: G. David Beiser, M.D.

Other Investigators: Stephen E. Epstein, M.D.
Morris Stampfer, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Others:	.4

Project Description: Although it is generally appreciated that the hemodynamic effects of exercise are influenced by changes in body position and the form of exercise performed, very little is known about the significance of these factors in patients with impaired cardiac function. Thus, data obtained from such patients during supine bicycle exercise, which is the method commonly in use, may not reflect accurately the circulatory responses occuring during ordinary upright activity. Accordingly, nine patients with moderate to severe cardiac impairment and eight subjects with normal cardiovascular systems were studied during both supine bicycle and upright treadmill exercise. At comparable levels of exertion, as determined by VO_2, there were no differences between the two modes of exercise in the response of cardiac index (CI) and stroke index (SI) in the patients. In normal subjects, however, CI was an average of 16% (0.83 L/min/M^2, $p < .005$) and SI 13% (7 ml/M^2, $p < .005$) lower during upright exercise. However, mean pulmonary arterial pressure (PAP) was lower in patients by an average of 17 mm Hg ($p < .005$) during upright exercise compared to a difference of only 5 mm Hg ($p < .05$) in normals. In patients heart rate (HR) was 10% (12 beats/min, $p < .05$) and arterial pressure 20% (20 mm Hg, $p < .001$) lower during upright exercise but they were unchanged in normals during comparable levels of exercise in the two positions.

Since the two types of exercise employed--that is, bicycle versus treadmill--might have also influenced the circulatory responses observed in the two positions, additional groups of eight normal subjects and eight patients were studied during upright exercise with both the bicycle and treadmill. At

'46

identical levels of oxygen uptake for each individual, heart rates and blood pressures were significantly higher in both groups during upright bicycle exercise than during treadmill exercise, suggesting that pedaling a bicycle is inherently more stressful than walking on a treadmill. When the same level of bicycle exercise was performed in both positions, heart rates were higher in the normal group during upright exercise. In contrast, heart rates and blood pressures were significantly lower in the patients during upright versus supine exercise on the bicycle. Thus exercise in the supine position appears to be more difficult for patients with cardiac impairment.

Proposed Course of Project: Project completed.

Honors and Awards: None

Publications: Manuscript in preparation.

Serial No.- NHI-35
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of Increased Wall Stress upon the Transmural Gradient of High-Energy Phosphates in Dog Myocardium

Previous Serial Number: None

Principal Investigator: Robert C. Boerth, M.D.

Other Investigators: James W. Covell, M.D.
John Ross, Jr., M.D.
Peter E. Pool, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: A transmural gradient of creatine phosphate (CP) has been reported to occur during experimental reduction of coronary blood flow; however, it is uncertain whether or not such a gradient exists under normal conditions, or when the workload of the heart is increased. In 6 canine left ventricular isovolumic preparations, peak wall stress (P_o) was increased from an average of 41 g/cm^2 to 72 g/cm^2 by augmenting left ventricular volume. Heart rate also was increased from an average of 131 to 204 beats per minute. Transmural biopsies were taken from the left ventricle and quickly frozen. The transmural biopsies were obtained using a modified dentist drill which had been adapted for this purpose. During the control state and the periods of increased cardiac workload, there was significantly more epicardial than endocardial CP (12.8 vs. 9.6 μmoles/g, $p < 0.05$); a small but significant drop in epicardial CP (13.4 to 11.6 μmoles/g) occurred when wall stress and heart rate were increased, but there were no changes in endocardial CP, or in the levels of ATP. It was concluded that: 1) there is normally a transmural gradient of CP, but 2) markedly increased wall stress can be sustained with only slight alteration in total energy stores and with no preferential lowering of endocardial high-energy phosphate levels.

Proposed Course of Project: The data collection and statistical analysis have been performed. The manuscript is now in preparation.

Honors and Awards: None
Publications: In preparation.

48

Serial No. - NHI-36
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of Increasing Heart Rate Upon Myocardial Oxygen Consumption

Previous Serial Number: None

Principal Investigator: Robert C. Boerth, M.D.

Other Investigators: James W. Covell, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years (computed for the 12 month period)

Total:	.8
Professional:	.4
Other:	.4

Project Description: It has been shown that wall stress and myocardial contractile state, as reflected in the peak stress (P_o) and maximum contractile element velocity ($_{max}V$) of the force-velocity relation, are major determinants of myocardial O_2 utilization ($M\dot{V}O_2$). Increasing heart rate is known to increase $_{max}V$; however, in previous studies relating heart rate to $M\dot{V}O_2$, P_o has not been controlled. In 8 isovolumic canine left ventricles, P_o was continuously calculated by an analog computer and held constant by altering ventricular volume, while heart rates were increased from 98 to 202 beats per minute. An increase in $_{max}V$ always accompanied the tachycardia. There was a significant linear regression of $M\dot{V}O_2$ on heart rate. In addition, there was a significant increase in $M\dot{V}O_2$ per beat with increasing heart rate and with the associated increase in $_{max}V$. These studies demonstrate that an increase in contractile state produced by an elevation of heart rate is accompanied by increased energy utilization, and they are in agreement with previous experiments showing a direct relationship between contractile state and $M\dot{V}O_2$.

Proposed Course of Project: Data collection and statistical evaluation are completed. A manuscript is now in preparation.

Honors and Awards: None

Publications: None

N7

Serial No.- NHI-37
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Association of Depressed Myofibrillar Adenosine Triphosphatase and Reduced Contractility in Experimental Heart Failure

Previous Serial Number: None

Principal Investigator: Brian M. Chandler, M.D.

Other Investigators: Peter E. Pool, M.D.
Edmund H. Sonnenblick, M.D.
James F. Spann, Jr., M.D.

Cooperating Units: None

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: Although previous studies have suggested a depression of myofibrillar ATPase in the presence of heart failure, their interpretation has been limited by failure to exclude mitochondrial contamination and lack of information regarding the contractility of the tissue studied. To explore this fundamental question, myofibrils were prepared from the right and left ventricles of 15 normal cats, 10 cats with right ventricular hypertrophy and 19 with right ventricular hypertrophy and right ventricular failure; graded pulmonary artery stenosis was produced in the latter groups. The contractility of these hearts was assessed using their right ventricular papillary muscles isolated in a myograph. Myofibrillar ATPase in all groups was maximally activated by 5mM ATP, 5 mM $MgCl_2$ at pH 7.0 and 37°C, while mitochondrial contamination was eliminated by sodium azide. In right ventricular failure, right ventricular myofibrillar ATPase was depressed by 39% from an average of 0.18 $\pm$ 0.01 in normal cats to 0.11 $\pm$ 0.01 μmole inorganic phosphate/mg protein per min ($P < .001$). In right ventricular hypertrophy, right ventricular myofibrillar ATPase was not significantly depressed (0.16 $\pm$ 0.01 μmole/mg per min). Of note, left ventricular myofibrillar ATPase was also significantly ($p < .05$) depressed in right ventricular failure from 0.16 $\pm$ 0.01 to 0.13 $\pm$ 0.01 μmole/mg per min. Contractility of the associated right ventricular papillary muscles, expressed as maximum rate of force development at the apex of the length-active tension curve, was correlated with myofibrillar ATPase activity.

150

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Proposed Course of Project: Project completed

Honors and Awards: None

Publications:

Chandler, B. M., Sonnenblick, E. H., Spann, J.F., Jr., and Pool, P.E.: The association of depressed myofibrillar adenosine triphosphatase and reduced contraction in experimental heart failure. Circulation Res. 21: 717-725, 1967.

Serial No. - NHI-38
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Influence of Homeometric Autoregulation on Myocardial Performance

Previous Serial Number: NHI - 11

Principal Investigator: Richard L. Clancy, Ph.D.

Other Investigators: Thomas P. Graham, Jr., M.D.
Edmund H. Sonnenblick, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: The intrinsic responses of the heart to an acute outflow resistance consist of an increase, followed by a smaller decrease in end-diastolic volume and pressure, a response termed hoemometric autoregulation. It has been suggested that this response is associated with an increase in contractility. Homeometric and heterometric autoregulation were produced in the in situ dog left ventricle, in which beta adrenergic receptors had been blocked with propranolol, by increasing aortic pressure and stroke volume respectively. Effects of instantaneous fiber length were controlled by comparing single isovolumic beats originating from similar end-diastolic circumferences, measured with a mercury-in-rubber gauge. The averages of 12 beats from 7 animals during homeometric autoregulation were: end-diastolic volume = 47.9 ml, stroke volume = 11.3 ml, aortic pressure = 113 mm Hg, and end-diastolic pressure 8.7 mm Hg. The corresponding values during heterometric autoregulation were: 47.2, 18.9, 78 and 9.9. During homeometric autoregulation average contractile element velocity and maximum isovolumic force exceeded the corresponding parameters during heterometric autoregulation by 10.9 and 7.4% respectively. The differences in contractile element velocity and maximum isovolumic force during heterometric and homeometric autoregulation in cardiac denervated animals were comparable to those observed in the animals given propranolol. It was concluded that the increase in myocardial performance during an acute increase in left ventricular outflow resistance is principally a result of the increased fiber length, an additional small increased in contractile state being attributed to homeometric autoregulation.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Proposed Course of Project: Project completed.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Clancy, R. L., Graham, T. P., Jr., Ross, J., Jr., Sonnenblic, E. H., and Braunwald, E.: The influence of aortic pressure-induced homeometric autoregulation on myocardial performance. Am. J. Physiol. In press.

Serial No.- NHI-39 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Chronic Beta Adrenergic Receptor Blockade for Idiopathic Hypertrophic Subaortic Stenosis

Previous Serial Number: NIH-15

Principal Investigator: Lawrence Sorel Cohen, M.D.

Other Investigators: Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: Exercise-induced angina pectoris is a common symptom in patients with idiopathic hypertrophic subaortic stenosis and at times is the major reason for their disability. Exercise is a potent stimulus to the augmentation of myocardial oxygen requirements as it activates cardiac adrenergic receptors and in patients with IHSS increases left ventricular outflow obstruction. The effect of beta adrenergic receptor blockade on the myocardial oxygen requirements of patients with IHSS are complex. Wall tension, heart rate, arterial pressure and velocity of contraction have all been demonstrated to be important determinants of myocardial oxygen requirements. Beta adrenergic blockade reduces the velocity of contraction, heart rate and probably wall tension. A diminution in all three of these would be expected to lower myocardial oxygen needs. On the other hand the negative chronotropic effect and diminution of sympathetic stimulation of the heart induced by beta adrenergic receptor blockade would tend to increase ventricular end-diastolic volume and by LaPlace's law to increase wall tension at any left ventricular systolic pressure.

In a previous study seven patients with angina pectoris were studied on an exercise treadmill. They received between 80 and 400 mg propranolol a day. Four of these seven patients exhibited significantly improved exercise tolerance on the days on which they received propranolol in comparison to the days on which placebo was administered. Subsequently an eighth patient has been studied and he too showed significant improvement with propranolol. Propranolol has been administered chronically to each of the patients who showed improvement. Two of the patients who initially responded to propranolol slowly became symptomatic on 160 mg. propranolol daily and each required operation at 18 months

and 19 months respectively after initiation of therapy. Two patients have maintained improvement for 18 and 29 months respectively but repeat exercise testing in one of them revealed a 30 per cent reduction in exercise tolerance. Of the three patients who initially showed little or no improvement on propranolol therapy, two have been operated upon with improvement in angina pectoris.

The present study suggests that in some patients with angina pectoris due to IHSS, propranolol causes amelioration of symptoms and may at least temporarily obviate the requirement for operation.

Proposed Course of Project: Project completed.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Cohen, L. S., and Braunwald, E.: Chronic Beta Adrenergic Receptor Blockade for Idiopathic Hypertrophic Subaortic Stenosis. Prog. in Cardiovasc. Dis. In press.

Serial No.- NHI-40
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Depression of Cardiac Function by Antimicrobial Agents.

Previous Serial Number: None

Principal Investigator: Lawrence Sorel Cohen, M.D.

Other Investigators: Gerald Glick, M.D.
Andrew S. Wechsler, M.D.

Cooperating Units: None

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: Although nephro-toxicity, neurotoxicity and ototoxicity are frequent concomitants of antibiotic therapy, direct cardiac toxicity has not been recognized. Interest in the present project grew out of the observation that persistent hypotension was a feature of the clinical course of a patient who manifested signs of streptomycin toxicity.

Work to date has confirmed the hypothesis that streptomycin has a depressor effect on cardiac function. Dose response curves measuring left ventricular dp/dt and right ventricular wall tension utilizing between 2.5 mg/kg to 40 mg/kg streptomycin intravenously revealed a 9 to 37% decline in LV dp/dt and a 12 to 42% decline in right ventricular wall tension. In the isolated perfused Langendorff cat heart preparation depression in isovolumetric pressure generation was observed when streptomycin was added to the perfusate.

Similar depressions in left ventricular function and in the isolated Langendorff preparation were seen when vancomycin, tetracycline, kanamycin and colymycin were tested. All of the agents were administered in doses commonly employed clinically.

Proposed Course of Project: Further work utilizing a right heart bypass preparation will be performed in order to define the site of primary action more precisely of these agents. In addition studies will be undertaken to define more precisely the mechanism by which streptomycin and other antimicrobial agents interfere with cardiac function.

Honors and Awards: None

Publications: None

Serial No.- NHI-41 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Heart Motion Video Tracking (Radarkymography) in the Diagnosis of Congenital and Acquired Heart Disease.

Previous Serial Number: NIH - 16 - (C)

Principal Investigator: Lawrence Sorel Cohen, M.D.

Other Investigators: Allan L. Simon, M.D.
Eugene Braunwald, M.D.

Cooperating Units: Department of Diagnostic Radiology
Clinical Center, NIH

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: A number of techniques for analyzing the motion of the cardiovascular silhouette are available. Fluoroscopy, the first method which attempted to evaluate both the size and motion of cardiac chambers relied upon the subjective impressions of the observer. Roentgenkymography lacked precision and displayed the borders of the cardiac silhouette only at random points throughout a cardiac cycle. The electrokymogram recorded directional changes in the movement of the heart border but the tracings were not linear and could not be quantified. The use of image intensification and closed circuit television during fluoroscopy has allowed the development of a more precise method for recording the movements of cardiovascular structures. Heart motion video tracking (Radarkymography) is such a technique and through its use motion of any cardiac border can be translated into a reproducible linear graphic tracing.

Over 100 patients with a variety of congenital and rheumatic heart lesions have been studied by radarkymography. Studies of the left ventricle delineated the motion of both the normal ventricle and the ventricles of patients with aneurysms. Movement of the left atrial wall in patients with mitral stenosis or regurgitation reflected the hemodynamic abnormalities characteristic of these conditions. Tracings from the aortic wall were obtained to define different varieties of left ventricular outflow tract obstruction. Coarctation of the aorta could be diagnosed by tracking the aorta proximal and distal to the coarctation.

Proposed Course of Project: Project completed.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Cohen, L. S., Simon, A. L., Whitehouse, W. C., Schuette, W. H., and Braunwald, E.: Heart Motion Video-Tracking (Radarkymography) in the Diagnosis of Congenital and Acquired Heart Disease. Am. J. Cardiol. In press.

Serial No. - NHI-42 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Clinical Spectrum of Mitral Regurgitation in Patients with Papillary Muscle Rupture or Dysfunction Due to Myocardial Infarction.

Previous Serial Number: None

Principal Investigator: Lawrence Sorel Cohen, M.D.

Other Investigators: Andrew G. Morrow, M.D.
Nina S. Braunwald, M.D.
William C. Roberts, M.D.
Eugene Braunwald, M.D.

Cooperating Units: Clinic of Surgery, NHI

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: Rupture of a left ventricular papillary muscle is an uncommon but well recognized complication of acute myocardial infarction. In the majority of patients, papillary muscle rupture is followed by death within hours. Other patients, in whom the performance of the left ventricle has been less seriously compromised by the infarct may survive for weeks or months, but will almost always develop congestive heart failure. Four patients with ruptured papillary muscles, severe mitral regurgitation and congestive heart failure were studied and operated upon. All were men, ranging in age from 51 to 69. Each was in severe congestive heart failure when first seen. The clinical examination was notable in that each of the patients was in sinus rhythm with diastolic filling and atrial gallop sounds. The electrocardiograms showed anterior infarction in one patient, inferior infarction in two patients and subendocardial infarction in one patient. Preoperative pulmonary artery systolic pressure ranged from 60 to 80 mm Hg in three patients. Pulmonary capillary wedge mean pressure ranged from 28-32 mm Hg in three patients with "v" waves 37 to 49 mm Hg. Cardiac index was subnormal in all. Each patient underwent replacement of the mitral valve with a Starr-Edwards prosthesis and each of them has been dramatically helped. Successful mitral valve replacement had been reported in only two patients with ruptured papillary muscles prior to this experience. Recently a patient with papillary muscle dysfunction, without rupture, whose clinical course was indistinguishable from the four patients with rupture has been operated upon with relief of symptoms.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Since the incidence of myocardial infarction is so very common, the syndrome of papillary muscle rupture or dysfunction is likely to be recognized with increasing frequency. If in a patient with severe disability following a myocardial infarction, catheterization studies indicate that the major reason for disability is mitral regurgitation, an increasing number of patients may be able to benefit from operative intervention.

Proposed Course of Project: Project completed

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Morrow, A. G., Cohen, L. S., Roberts, W. C., Braunwald, N. S., and Braunwald, E.: Severe mitral regurgitation following acute myocardial infarction and ruptured papillary muscle. Circulation. Suppl. II, 37-38: II-124, 1968.

160

Serial No.- NHI-43

1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Congestive Heart Failure Following Chronic Tachycardia. Characterization of Left Ventricular Contractility and Energy Stores.

Previous Serial Number: None

Principal Investigator: Henry N. Coleman, M.D.

Other Investigators: Roger R. Taylor, M.D.
Peter E. Pool, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: Intensive investigation of the syndrome of chronic congestive heart failure (CHF) has resulted in delineation of alterations in function or organ systems, the autonomic nervous system, and the peripheral circulation. However, limitations imposed by assessing cardiac function in hemodynamic terms have made it difficult to define the contractile state during CHF. With development of methods allowing characterization of the mechanics of myocardial contraction, it is now feasible to define the contractile state of the myocardium of the chronically failing heart in quantitative terms. Accordingly, CHF was induced in 6 surgically prepared dogs by 2-4 weeks of pacemaker induced chronic ventricular tachycardia at 280 contractions/min. Twenty-four hours following cessation of stimulation contractile element velocity and wall tension were calculated for isovolumic left ventricular contractions obtained from intact sedated dogs. Then, following anesthesia, transmural left ventricular biopsies were obtained for determination of myocardial norepinephrine, creatine, creatine phosphate, and ATP in the 6 animals with CHF and 8 sham-operated control animals.

The results demonstrate a highly significant depression of the contractile state, characterized by marked depression of the intrinsic velocity of contraction and active isovolumic tension, in the animals with CHF produced by chronic tachycardia. In addition, total myocardial energy stores are significantly depressed; myocardial norepinephrine was depressed from normal in both the CHF and sham-operated control animals.

161

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Proposed Course of Project: Experiments have been completed and manuscript is in preparation.

Honors and Awards: None

Publications: None

Serial No. - NHI-44
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Norepinephrine and Myocardial Oxygen Consumption.

Previous Serial Number: None

Principal Investigator: Henry N. Coleman, M.D.

Other Investigators: Edmund H. Sonnenblick, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: In addition to the effects of tension development and external work on myocardial oxygen consumption ($M\dot{V}O_2$), recent studies have suggested that the velocity of contraction is an important determinant of $M\dot{V}O_2$. Of particular interest among the agents capable of augmenting the velocity of contraction are the effects of the physiologic sympathetic neurotransmitter, norepinephrine. Although recent work from this laboratory implicated norepinephrine induced changes in the velocity of contraction as a determinant of $M\dot{V}O_2$, the influence of concomitant alterations in shortening and external work prevented a quantitative examination of the relation of velocity of contraction to $M\dot{V}O_2$.

The objective of the present study was to evaluate the relationship between norepinephrine induced changes in intrinsic velocity of contraction and $M\dot{V}O_2$. In order to isolate a single variable, comparison of isometric contractions prior to and following a variety of concentrations of norepinephrine at a constant level of tension development was accomplished. The results indicate that norepinephrine-induced augmentation of the contractile state, characterized by increased intrinsic velocity of contraction, results in an increased $M\dot{V}O_2$. At a constant level of tension development, the increments in $M\dot{V}O_2$ were found to be directly proportional to the degree of augmentation of the contractile state.

Proposed Course of Project: Experiments have been completed and the manuscript is in preparation.

Honors and Awards: None

Publications: None

Serial No.- NHI-45
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Description and Quantification of the Effects of Alterations in Tension, Shortening, and External Work on Myocardial Oxygen Consumption.

Previous Serial Number: NHI - 20

Principal Investigator: Henry N. Coleman, M.D.

Other Investigators: Edmund H. Sonnenblick, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: The development of tension has long been recognized as an important determinant of energy utilization of both skeletal and cardiac muscle. In addition, Fenn demonstrated in skeletal muscle that the onset of shortening against a load results in an additional energy utilization which is proportional to the work done. The effect of external work on the oxygen consumption of heart muscle (MVO_2) was described in qualitative terms in the initial phase of this project.

Quantification of the effect of tension development and external work was accomplished by comparing the MVO_2 of isometric contractions to those of afterloaded contractions at a constant level of tension development in 14 additional muscles. The performance of shortening and external work in afterloaded contractions was associated with an additional oxygen consumption which was directly proportional to the external work performed. At one half isometric load the performance of external work was found to account for 48% of the total $\dot{M}VO_2$.

Evaluation of the unit energy cost of performing external work and comparison to the unit energy cost of work performed in tension development through extending the series elastic component ["internal contractile element work" (CEW)] revealed that the unit energy cost of "internal CEW" was 2 times the unit energy cost of performing external work. Since the concept of CEW as a determinant of $\dot{M}VO_2$ is based on the assumption that the unit energy cost of work is independent of the manner in which it is performed, these results

indicate that CEW is not a simple, linear determinant of $M\dot{V}O_2$. Comparison of calculated CEW to $M\dot{V}O_2$ verified this prediction.

Proposed Course of Project: Project completed. Results from initial phase have been published and manuscript for the second phase is being prepared.

Honors and Awards: None

ARTICLE PUBLISHED IN PERIODICAL:

Coleman, H. N.: Effect of alterations in shortening and external work on oxygen consumption of cat papillary muscle. Am. J. Physiol. 214: 100, 1968.

Serial No. - NHI-46
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Role of Calcium and Heart Rate in Increasing Myocardial Oxygen Consumption.

Previous Serial Number: NHI - 19

Principal Investigator: Henry N. Coleman, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	.6
Professional:	.3
Other:	.3

Project Description: Although increments in calcium ion concentration and the rate of stimulation have been demonstrated to augment the contractile state of the heart, the effect of these agents on myocardial oxygen consumption ($M\dot{V}O_2$) has not been clearly defined. However, recent studies in this laboratory have implicated the velocity of contraction as a determinant of $M\dot{V}O_2$. Since both of these interventions have been demonstrated to augment the intrinsic velocity of cat papillary muscle, it appeared desirable to determine the effect of these agents on $M\dot{V}O_2$ of isolated cat papillary muscle. Accordingly, the effect of variation in frequency of stimulation from 15 to 60/min on myocardial mechanics and $M\dot{V}O_2$ was studied in 8 preparations. Augmentation of the contractile state resulted in alterations in both velocity of contraction and shortening and external work. These changes in mechanical behavior were in turn associated with an increased $M\dot{V}O_2$. Augmentation of the contractile state with Ca^{++} ion produced similar changes in mechanical behavior which were in turn associated with an increased $M\dot{V}O_2$.

Proposed Course of Project: Since the present experiments do not allow separation of the effects on $M\dot{V}O_2$ of increments in velocity of contraction from changes in external work, additional experiments are planned. In these experiments the $M\dot{V}O_2$ of isometric contractions at a constant level of tension development will be determined prior to and following augmentation of the contractile state will allow description and quantitation of the effects of alterations in contractile state on $M\dot{V}O_2$.

Honors and Awards: None

Publications: None

Serial No. - NHI-47
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Series Elasticity in the Intact Left Ventricle Determined by a Quick Release Technique.

Previous Serial Number: NIH-24

Principal Investigator: James W. Covell, M.D.

Other Investigators: Roger R. Taylor, M.B., MRACP
Edmund H. Sonnenblick, M.D.
John Ross, Jr., M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: In isolated muscle, the series elastic component (SEC) can be analyzed by determining the length changes following quick releases to known loads during contraction. The characteristics of the effective SEC of the intact left ventricle (LV) were determined by a quick release method in 11 dogs in which the LV contracted isovolumically against a balloon inserted through the mitral annulus. During active contraction, sequential withdrawals of 0.5 to 7.0 ml of fluid were performed rapidly (5-18 msec) by an electrically triggered mechanical syringe. The calculated reductions of the LV midwall circumference were plotted against the corresponding changes in mean wall stress ($T = PR/2h$, P = LV pressure, R = internal LV radius, h = wall thickness). The resulting load-extension curves were exponential, and the reciprocals of their slopes (dT/dl) were linearly related to T: $dT/dl = (26.8 \pm 2.2, \text{SEM}) \times T + 808 \pm 78$. The maximum extension of the SEC averaged 4.36% of LV circumference (range 2.63 to 5.49%) at LV pressures averaging 79/6 mm Hg (systolic/end-diastolic; range 66-107/0-15 mm Hg). The load-extension curve was unchanged by varying the time of release and by norepinephrine infusion. The data were also examined for ellipsoidal and two spherical models for the calculation of wall stress and there are no qualitative differences in the shape of the load extension curves thus obtained. These data support a model for the intact LV that contains an undamped SEC, the characteristics of which resemble those of isolated cardiac muscle.

Proposed Course of Project: Study complete.

167

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Honors and Awards: None

Publications: Manuscript in preparation.

Serial No. - NHI-48

1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A technique for the production of isotonic contractions in the intact canine heart.

Previous Serial Number: None

Principal Investigator: James W. Covell, M.D.

Other Investigators: Robert Boerth, M.D.
John Ross, Jr., M.D.
Jack Fuhrer

Cooperating Units: Biomedical Engineering and Instrumentation Branch

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: The objective of this study was to develop a device capable of controlling calculated myocardial wall stress in the intact canine left ventricle. This system would then allow the direct examination of contractile element activity in the intact heart. The system devised consists of a DC driving motor which is connected to a piston and cylinder arrangement. The cylinder is connected to a glass cannula and latex balloon which is sutured into the mitral valve with the latex balloon in the ventricular cavity. Ventricular volume is thus regulated by the driving motor. The animal is maintained on total cardiopulmonary bypass. Myocardial wall stress is calculated from intraventricular pressure and ventricular volume on an analog computer, and a tension error signal (control level - calculated tension) is generated. This signal is used to drive the motor, regulate ventricular volume and thus control wall tension. In four preliminary experiments the device has functioned satisfactorily. Wall stress control has been within 5% of the control level throughout contraction. Under these conditions as the stress control level is raised the extent of fiber shortening decreases and the initial velocity of shortening is an inverse function of load.

Proposed Course of Project: Further experiments are planned.

Honors and Awards: None

Publications: None

Serial No. - NHI-49 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Post-operative Psychosis in Non-English Speaking Patients

Previous Serial Number: None

Principal Investigator: Delores A. Danilowicz, M.D.

Other Investigators: None

Cooperating Units: Surgery Branch, National Heart Institute

Man Years:

Total:	.6
Professional:	.3
Others:	.3

Project Description: The occurrence of acute psychosis in patients having open heart operations has been noted in several series. This is usually a transient episode occurring in the recovery room and has been credited to the absence of REM sleep, the possible effects of extra-corporeal circulation, and the isolation of the patient from normal interpersonal relationships. If a large part of this psychosis were dependent on contact with reality through interpersonal relationships, then patients not speaking English would be subjected to a much more severe degree of isolation and would be more prone to the development of this acute psychosis.

Fifty-five (55) adult patients (over 15 years of age), not speaking English and undergoing open heart surgery were evaluated in retrospect for acute psychosis and other behavioral disorders. Of 32 males, 14 had acute psychoses and 6 had behavioral disturbances noteworthy enough to be mentioned in the nurses' or doctors' notes. Of 23 females, 2 had acute psychoses and 7 had "minor" behavioral disturbances. There were 14 and 8 major medical complications in the male and female groups and death occurred in 5 and 3 instances respectively.

A control group consisting of English speaking patients usually from the United States was analyzed. Selection was directed at attempting an approximate matching of age and type of heart disease. Only a small part of this control group has been reviewed to date--11 males and 15 females--but even in this series, there have been no psychoses and only 3 "abnormal" behavioral patterns, 1 in the male group and 2 in the female group. Further data are to be gathered to see if this difference persists when a larger control group is compared. It seems, however, that the rate of acute psychosis in the

170

post-operative period is higher in the non-English speaking group. Complication and death rate will also be compared in the two groups.

Proposed Course of Project: To be completed.

Honors and Awards: None

Publications: None

Serial No. - NHI-50 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Retarded Bone Age in Children with Cyanotic Congenital Heart Disease

Previous Serial Number: NHI-25(c)

Principal Investigator: Delores A. Danilowicz, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	.6
Professional:	.3
Others:	.3

Project Description: The mechanisms responsible for growth retardation in patients with congenital heart disease remain obscure. In the present investigation, the relationship between bone age, roentgenographically determined, and the systemic arterial oxygen saturation, measured at cardiac catheterization, was examined in 110 children with congenital heart disease. Forty-nine of the patients had acyanotic heart disease and 61 had varying degrees of desaturation of the arterial blood. Only 3 (6%) of the children in the acyanotic group showed bone age retardation. In the cyanotic groups, however, 22/61 were found to have retardation of bone age. A highly significant positive correlation existed in the cyanotic patients between the ratio of bone age to chronological age and the resting systemic arterial oxygen saturation ($r = .733$). After successfulcorrective or palliative operations in three of the children with cyanotic heart disease and retarded bone ages, all exhibited an accelerated bone maturation. Detailed studies of endocrine and renal function, serum proteins, and chromosome karyotypes in the cyanotic patients were normal. Whether or not the close correlation observed between blood oxygen saturation and bone age can be explained by the effects of hypoxemia per se on bone metabolism remains open to speculation.

Proposed Course of Project: Collection of more data will continue. The role of growth hormone may be investigated.

Honors and Awards: None

Publications: None

Serial No. NHI-51
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Pulmonary Resistance in the Large Pulmonary Arteries in the Newborn Lamb

Previous Serial Number: NHI-26

Principal Investigator: Delores A. Danilowicz, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	.6
Professional:	.3
Others:	.3

Project Description: It has been observed at cardiac catheterization that many newborn infants have a pressure gradient between the main peripheral pulmonary arteries. These gradients are at times large enough to cause significant differences in the calculated pulmonary resistance when calculated using main and peripheral mean pulmonary artery pressures. On recatheterization of several of these children at an older age, this gradient was no longer present. This was thought, therefore, to represent a normal physiological change secondary to growth of the peripheral pulmonary artery branches, which are relatively hypoplastic at birth. Since most of the children in whom these observations were made had some type of congenital cardiac defect, it was of interest to determine whether this gradient exists in otherwise normal hearts and to measure the pressures without a catheter obstructing the vessel in which the pressure measurement was being made.

Newborn lambs were used as the experimental animal in an attempt to confirm the presence of a gradient from main to peripheral pulmonary artery. Thoracotomy was performed in 18 animals ranging from 12 hours to 4 months in age. Pressures were measured in main and left pulmonary artery using a matched system. In seven of the 18 animals, certain interventions were added to study the change in the gradient. Systolic gradients at the bifurcation of the pulmonary artery were found in 15 of the 16 animals under 18 days of age (range = 2-12 mm Hg; average = 6 mm Hg). Mean pressure gradients were limited mainly to the animals under 48 hours of age. Although the gradients disappeared with age, they could be induced in the older animals by increasing flow or by increasing cardiac contractility. By the age of 4 months, however, even these interventions could not create a gradient. Silicone casts of the

right ventricle and pulmonary artery tree showed two major changes with age: 1) Growth of the peripheral branches and 2) a lessening of the angulation between the main and peripheral branches.

The presence of this gradient adds another reason for the elevation of pulmonary vascular resistance immediately after birth especially if calculation is based on right ventricular or main pulmonary arterial pressures. It may be important in infants with congenital heart lesions causing large left to right shunts for special efforts to be made to measure the pressure in the peripheral pulmonary artery since it may be significantly lower than main pulmonary arterial pressure.

Proposed Course of Project: Project completed.

Honors and Awards: None

Publications: Manuscript in preparation.

174

Serial No. - NHI-52
1. Cardiology
2. Pharmacology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Supersensitivity of the Chronically Denervated Feline Heart

Previous Serial Number: None

Principal Investigator: Peter J. Dempsey, M.D.

Other Investigators: Theodore Cooper, M.D., Ph.D.

Cooperating Units: None

Man Years:

Total:	.6
Professional:	.3
Other:	.3

Project Description: It has been shown that the extrinsically denervated feline heart manifests a "supersensitivity" to exogenous norepinephrine. Although the general phenomenon of denervation supersensitivity has been observed in virtually all systems of the body, the precise mechanisms underlying denervation supersensitivity of the heart have not been established. In order to study the role that the postjunctional receptor site changes, as well as the loss of binding sites for catecholamines might play, the responses of the denervated heart to norepinephrine were compared with the responses of the same hearts to isoproterenol and calcium. The peak tension developed in the isovolumically beating left ventricle of isolated perfused hearts was used as an index of contractility.

Cardiac denervation resulted in a significant lowering of the threshold dose of norepinephrine and a shift of the dose response curve upwards and to the left. Although there was no change in the threshold dose of isoproterenol, there was a significant reduction in responsiveness at higher doses. No changes were noted with calcium.

These data give no evidence of a proliferation of beta receptor sites or of nonspecific effector membrane changes in the chronically denervated heart. The "supersensitivity" can be explained by a loss of intraneuronal binding loci for norepinephrine.

Proposed Course of Project: Although the original project stands as completed, the presence of intact nerve cell bodies and axons after extrinsic denervation and their possible effector cell membrane role still remains a problem. Further studies concerning enzyme and electrical changes in cells

after denervation are in progress.

Honors and Awards: None

Publications:

Dempsey, P. J., and Cooper, T.: Supersensitivity of the chronically denervated feline heart. Am. J. Physiol. Submitted for publication.

Serial No. NHI-53
1. Cardiology
2. Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Ventricular Receptors for Acetylcholine: Incidence for Nicotinic and Muscarinic Sites

Previous Serial Number: None

Principal Investigator: Peter J. Dempsey, M.D.

Other Investigators: Theodore Cooper, M.D., Ph.D.

Cooperating Units: None

Man Years
Total: .6
Professional: .3
Other: .3

Project Description: It has been demonstrated in an isolated heart preparation that "nicotinic" doses of acetylcholine will release norepinephrine from binding sites. It has also been reported, however, that the positive inotropic effects of acetylcholine on slowly beating cat papillary muscles may be independent of catecholamine mediated mechanisms. Therefore, in an attempt to demonstrate dual positive inotropic mechanisms (catecholamine dependent and independent), the acetylcholine responses of normal and catecholamine depleted (by both surgical and pharmacologic means) feline hearts were compared by an isolated perfusion technique.

It has been observed that acetylcholine has both negative and positive inotropic effects on the intact ventricle. The negative effects appear to be mediated by muscarinic receptors (blocked by atropine) which are located on the muscle cells. The positive effects appear to be catecholamine dependent (blocked by propranolol and catecholamine depletion) and mediated by "nicotinic" receptors (blocked by d-tubocurarine).

Proposed Course of Project: Three major areas will be examined in the light of the above findings: First, the alteration in the acetylcholine responses of normal and catecholamine depleted hearts after anticholinesterase pretreatment; secondly, the effects of cocaine on the magnitude of the observed responses; and thirdly, the effect of altering the extracellular $[Na^+]$ and $[Ca^{++}]$. Such studies should further separate the two effects of acetylcholine, and indirectly provide more information on the mechanism of action of the chronically denervated heart.

Honors and Awards: None

Publications: None

Serial No. - NHI-54
1. Cardiology
2. Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Atrial and Ventricular Intracellular Action Potentials in the Normal and Extrinsically Denervated Feline Heart

Previous Serial Number: None

Principal Investigator: Peter J. Dempsey, M.D.

Other Investigators: Theodore Cooper, M.D., Ph.D.

Cooperating Units: None

Man Years

Total:	.6
Professional:	.3
Other:	.3

Project Description: It is known that the extrinsically denervated feline heart manifests a supersensitivity to exogenous norepinephrine. It is also known that in structures with well delineated neuro-effector functions, such as skeletal muscle, definite postjunctional membrane changes occur following denervation.

Inasmuch as the nature of the terminal innervation of the heart still remains obscure, and as preliminary pharmacologic studies have not revealed evidence of such a postjunctional change, it was thought that study of cardiac muscle cells in the extrinsically denervated feline heart by electrophysiologic techniques might afford information about their functional state unobtainable by other means.

Intracellular action potentials have been recorded in atrial and ventricular tissue from both normal and denervated hearts using stand KCl glass electrodes. Preliminary results seems to show a change in phase 2 of the denervated ventricular action potential.

Proposed Course of Project: More of the same experiments will be carried out to establish firmly the reproducibility of our observation. Another set of experiments is then planned to compare the magnitude of changes on the action potential to norepinephrine added to the bath in normal and denervated hearts. Finally, action potentials will be recorded from hearts depleted of norepinephrine by reserpine rather than by surgery.

Honors and Awards: None
Publications: None

178

Serial No. - NHI-55
1. Cardiology
2. Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Intracellular Action Potentials in the Muscle Cells of the Mitral Valve

Previous Serial Number: None

Principal Investigator: Peter J. Dempsey, M.D.

Other Investigators: Theodore Cooper, M.D., Ph.D.

Cooperating Units: None

Man Years

Total:	.6
Professional:	.3
Other:	.3

Project Description: The anterior leaflet of the mitral valve has been shown to contain blood vessels, nerve fibers, and cardiac muscle in addition to elastic fibers and collagen. When studied in a myograph, it actively developed tension and shortened. This tension was increased by norepinephrine and decreased by acetylcholine. In situ surface electrocardiograms have been recorded corresponding in time to ventricular activation. It was felt, therefore, that by establishing the wave form of the intracellular action potential in the mitral valve muscle cells, it could be determined whether their activation was atrial or ventricular.

Due mainly to the technical problems encountered with such large amounts of connective tissue, only two recordings have been obtained thus far. These both, however, appear to possess a typical ventricular action potential wave form, suggesting that this muscle is fundamentally ventricular in nature.

Proposed Course of Project: Work will continue in trying to solve the technical problems of recording in this peculiar tissue. Perhaps new types of electrodes will have to be tried in the future.

Another possibility being considered is in studying very young animals with a more favorable muscle cell/connective tissue ratio.

Honors and Awards: None

Publications: None

Serial No.- NHI-56
1. Cardiology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Acetylcholine and Norepinephrine Interactions on the Inotropic State of Ventricular Myocardium

Previous Serial Number: None

Principal Investigator: Peter J. Dempsey, M.D.

Other Investigators: Theodore Cooper, M.D., Ph.D.

Cooperating Units: None

Man Years:

Total:	.6
Professional:	.3
Other:	.3

Project Description: Although it has been shown that acetylcholine in "nicotinic" doses will cause a catecholamine mediated positive inotropic effect on ventricular myocardium, it has also been shown that acetylcholine will actually antagonize various other catecholamine mediated effects on the heart. Mechanisms underlying these apparently incongruous cardiac effects of acetylcholine were examined in both normal and catecholamine depleted (by both surgical and pharmacologic means) feline hearts using an isolated perfusion technique.

These studies showed that acetylcholine has both negative (direct) and positive (indirect) inotropic effects on the intact ventricle. They also confirmed the previous reports of an acetylcholine-norepinephrine antagonism, and indicated that the indirect effect (or "nicotinic" action) of acetylcholine does not appear to be involved in the antagonism. Further, there was evidence that the chemical effects of acetylcholine on the ventricular muscle receptors may be temporally dissociated from the expressions of the drug effect on contractility.

Proposed Course of Project: The latter observation in these preliminary results has pointed up the need to document to a much more precise degree the time course of all the interventions studied. Further, since the adenyl cyclase system has been implicated in this phenomenon by previous investigators, direct chemical analysis of this enzyme system in denervated tissue is critical. All these answers would hopefully result in an understanding of not only the nature of the interaction of two chemical substances, but more importantly of the interaction in the heart of the sympathetic and parasympathetic nervous system.

PHS-NIH
Individual Projgect Report
July 1, 1967 through June 30, 1968

Honors and Awards: None

Publications: None

Serial No. -NHI-57 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effects of Pectus Excavatum on the Circulatory Response to Intense Exercise

Previous Serial Number: NHI - 32 (c)

Principal Investigator: Stephen E. Epstein, M.D.

Other Investigators: G. David Beiser, M.D.
Morris Stampfer, M.D.

Cooperating Units: None

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: Although much has been written concerning both the cosmetic and physical benefits to be derived from surgical correction of a pectus excavatum chest deformity, all attempts to identify a hemodynamic abnormality have failed. As a result of this, it is the current feeling of most clinicians that the entity of "pectus heart disease" does not in fact exist.

However, the observation that marked tachycardia developed in one such patient while standing, suggested to us that an impairment of cardiac function might exist only in the upright position, when the heart descends into that area of the thoracic cavity which is most narrowed by the chest wall deformity. Relevant to this is the fact that most previous hemodynamic studies of patients with pectus excavatum in which no abnormalities were demonstrated were performed in the supine position. In addition, on the basis of previous studies from this laboratory, it was felt that the most sensitive way to evaluate cardiac function in patients with equivocal cardiac impairment would be to measure cardiac output during maximal upright exercise.

Accordingly, 6 patients with mild to moderate degrees of pectus excavatum were studied at rest and during mild exercise in the supine position, and then at rest and during intense exercise in the upright position. Mixed venous O_2 content and pulmonary arterial pressure were obtained through a catheter previously introduced in the pulmonary artery during right heart catheterization. Arterial pressure and O_2 content were obtained through a

182

teflon catheter introduced percutaneously into the brachial artery. Cardiac output was measured by the Fick principle.

Right heart catheterization was normal in each of the patients as was the response to supine exercise. However, two patients demonstrated a definite impairment of the cardiac output to intense upright exercise.

Of the six patients, two had operative repair of the pectus deformity; one had an abnormal cardiac output response to intense upright exercise, and the second fell within the lower limits of normal. Several months post-operatively a repeat study in each patient demonstrated a significant increase in the response.

Proposed Course of Project: The purpose of future studies will be to determine the incidence of cardiac impairment in a larger number of such patients, to determine the degree of chest wall deformity necessary to produce cardiac impairment, and to evaluate the effects of corrective surgery in a greater number of patients.

Honors and Awards: None

Publications: None

Serial No.- NHI-58 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of a Cold Environment on the Circulatory Reponse to Exercise: Implications Concerning Angina Pectoris

Previous Serial Number: NHI - 29 (c)

Principal Investigator: Stephen E. Epstein, M.D.

Other Investigators: Morris Stampfer, M.D.
G. David Beiser, M.D.
Robert E. Goldstein, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total: .8
Professional: .4
Other: .4

Project Description: Clinical observers have long recognized that angina pectoris occurs more readily when exertion is performed in a cold rather than in a warm environment. To elucidate the physiologic basis for this observation 6 patients with angina due to coronary artery disease (CAD) and 5 subjects without CAD with either mild or no impairment of cardiac function were studied at rest and during the same level of mild upright exercise at 2 ambient temperatures: 23°C and 15°C. Results were similar in both groups of subjects. Changing from warm to cold did not alter cardiac output at rest (4.47 L/min vs. 4.50) or during exercise (7.10 vs. 7.26), heart rate at rest (90 vs. 91) or during exercise (101 vs. 100). However, at the lower temperature significant ($p < .05$) changes occurred in the following variables: mean systemic arterial pressure increased from 92 to 105 mm Hg at rest and from 92 to 110 during exercise; total peripheral resistance increased from 1609 to 1822 dyne sec.cm^{-5} at rest and from 993 to 1213 during exercise; left ventricular minute work increased from 5.7 to 6.5 kg-m at rest and from 9.0 to 10.9 during exercise. These results indicate that a cold environment provokes an increase in total peripheral resistance at rest and during exercise. The consequent rise in arterial pressure, by augmenting myocardial O_2 requirements, would thus contribute to an earlier onset of angina.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: Manuscript in preparation.

154

Serial No. - NHI-59
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Relationship Between Vitamin D and the Cranio-Facial and Dental Anomalies of the Supravalvular Aortic Stenosis Syndrome.

Previous Serial Number: NIH-36

Principal Investigator: William F. Friedman, M.D.

Other Investigators: Loren F. Mills, D.D.S.

Cooperating Unit: National Institute of Dental Research

Man Years:

Total:	.6
Professional:	.3
Other:	.3

Project Description: Recent investigations have suggested that a derangement in vitamin D metabolism during pregnancy may be responsible for the cardiovascular anomalies of the supravalvular aortic stenosis syndrome (SASS), especially when the latter is associated with idiopathic infantile hypercalcemia. In addition to the aortic lesion, prominent features of SASS include mental retardation, strabismus, a peculiar "elfin" facies and dental abnormalities, especially malocclusion. The present study was designed to explore the relationship between experimentally induced hypervitaminosis D in the mother and the development in the offspring of the cranio-facial and dental abnormalities observed in SASS.

Fifteen pregnant, white New Zealand rabbits, designated "test" animals, were fed a stock diet and given divided doses of intramuscular vitamin D_2 (activated ergosterol in cotton seed oil) every other day, starting on the second day after insemination and continuing until delivery. The total dose administered was 750,000 units. The offspring of six pregnant females who received injections of the cotton seed oil only and of fifteen unmolested pregnant females served as controls. When a test offspring was sacrificed or died spontaneously, a rabbit of the same size born to a control mother and selected by a random number technique was sacrificed. Each animal was decapitated, and several days fixation in 80% ethyl alcohol, the soft tissues were removed to expose the bony cranio-facial complex. A 3-dimensional orthodontic calipers was employed for measuring dental arch dimensions, and radiographs, casts, and histological sections of the skull and jaws were examined.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

The external appearance, the contour of the cranio-facial bones, and the dental anatomy were remarkably similar in all control offspring. The average weight of each test newborn was significantly less than control offspring ($P<0.01$), as was the crown-rump length ($P<0.01$). Characteristic malformations were noted upon exposure of the bony parts of the test skulls. Premature closure of the saggital, coronal, and lambdoidal sutures was a constant finding and provided the most striking difference in the external appearance of the skull when compared with control offspring. The skulls of the test animals, matched chronologically or by equivalent size with the controls, were smaller in the anterio-posterior plane and more narrow in the coronal plane. The body of the mandible was narrower, the angle of the ramus more obtuse, and the premaxillary and nasal bones smaller and somewhat compressed in appearance. The paired nasal bones displayed bossing near their posterior borders and the paired frontal bones exhibited marked bossing slightly posterior to the supraorbital ridges. The paired parietal bones appeared flattened in test animals, creating a level, rather than a raised and rounded appearance posteriorly. Caudally, the occipital bones were smaller and their nuchal surface rougher when compared to controls. The external occipital protuberance was particularly prominent in test offspring. Additional abnormalities that were noted in the test offspring included strabismus, buphthalmos, and shorter, occasionally notched ears.

Striking differences in dentition were noted between the groups. In the test sample, the maxillary and mandibular central incisors exhibited severe enamel hypoplasia of the incisal one-third of the tooth. This finding occurred in 95% of the test progeny. A prominent anterior crossbite was noted in 66% of the test offspring.

These experimental observations suggest that in SASS the cranio-facial and dental pecularities, as well as the aortic lesion, may be related to a derangement in vitamin D metabolism during pregnancy. Moreover, the ease of recognition and the higher incidence of skeletal and orthodontic malformations (70-95%) than cardiovascular lesions (40%) in test neonates indicates that the former findings may be the most useful markers for future testing of vitamin D teratogenicity.

Proposed Course of Project: Project completed.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Friedman, W. F., and Mills, L. F.: The relationship between vitamin D and the cranio-facial and dental anomalies of the supravalvular aortic stenosis syndrome. Pediatrics. In press.

Serial No.- NHI-60
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of frequency of contraction and Calcium Concentration on the Cardiac Response to Acetylcholine

Previous Serial Number: NIH-39

Principal Investigator: William F. Friedman, M.D.

Other Investigators: Robert A. Buccino, M.D.
Edmund H. Sonnenblick, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: In an earlier study it was suggested that myocardial tension development is reduced when muscarinic receptors in either atrial or ventricular myocardium are stimulated by low concentrations (1 μg/ml or less) of acetylcholine (ACh). These receptors are thought to be associated with vagal nerve endings and to be present in much greater concentration in atrial than in ventricular myocardium. When relatively high concentrations (30 to 300 μg/ml) of ACh are employed, a positive inotropic effect can be elicited from ventricular myocardium. ACh is known to release norepinephrine from sympathetic nerve endings in the heart and it has been suggested that the stimulation of ventricular contractility by ACh results entirely from the release of norepinephrine. However, this now appears to be unlikely, since the augmentation of tension development exerted by high concentrations of ACh may also occur in the absence of sympathetic innervation and intact ventricular norepinephrine stores.

In the present study the effects of frequency of contraction and of external calcium and sodium concentration on the inotropic actions of acetylcholine on isolated mammalian myocardium were examined. In stimulated left atria of cats, ACh reduced tension development, i.e., exerted a negative inotropic effect, which became more striking as the frequency of contraction was increased and which was inhibited by atropine. In cat papillary muscles studied at a low frequency (< 42/min), high concentrations of ACh augmented tension development, i.e., produced a positive inotropic effect, which was enhanced by atropine. At high frequencies

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

(< 72/min) this positive effect of ACh was abolished. Moreover, at high frequencies, ACh consistently reduced the normal increment in tension associated with increasing frequency. The magnitude of the positive inotropic action of ACh on papillary muscle was abolished by lowering the extracellular $[Na^{+}]$ and varied inversely with the extracellular $[Ca^{++}]$.

These findings were interpreted in terms of a dual action of ACh on the myocardium: (1) an action on the muscarinic receptor that reduced contractility, and (2) a direct effect on cellular membrane permeability to Ca^{++} that stimulates contractility. The first action may be blocked by atropine, while the second may be interfered with, or even prevented, by prior augmentation of intracytoplasmic $[Ca^{++}]$. The activation of the large number of muscarinic receptors in atrial myocardium by ACh depresses atrial contractility, particularly at high frequencies of contraction. In ventricular myocardium the muscarinic effect is relatively small, and ACh augments contractility unless intracytoplasmic $[Ca^{++}]$ has already been elevated. If it has been, the muscarinic effect of ACh is unopposed, resulting in a decrease in contractility which may be blocked by atropine.

Proposed Course of Project: Project completed.

Honors and Awards: None

Publications

ARTICLE PUBLISHED IN A PERIODICAL:

Friedman, W. F., Buccino, R. A., Sonnenblick, E. H., and Braunwald, E.: The effects of frequency of contractions and ionic environment on the responses of myocardium to acetylcholine. Circulation Res. 21: 573, 1967.

Serial No.-NHI-61
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Intrinsic Mechanical Properties of the Myocardium in the Developing Mammal.

Previous Serial Number: NIH-37

Principal Investigator: William F. Friedman, M.D.

Other Investigators: Charles Cooper, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: There are no observations available on the intrinsic mechanical properties of isolated fetal and neonatal heart muscle. Although several lines of evidence suggest that certain structural and chemical properties of heart muscle may be age-dependent, many difficulties have surrounded previous efforts to analyze the intrinsic mechanical properties of the fetal heart in situ. These problems have arisen largely because of the complex relations between the maternal and fetal circulations, and differences in the intra-uterine and delivery experiences. Moreover, adequate techniques have not been available for assessing quantitatively the function of the intact fetal or newborn heart. An attempt has been made in the present investigation to obviate these problems by studying isolated cardiac tissue obtained from the hearts of animals of several species during the fetal and neonatal period. The intrinsic mechanical properties of the developing muscle are analyzed in a myograph and compared with those of adult atria and papillary muscles studied under identical conditions.

From the hearts of fetal, neonatal, and adult lambs and swine, isolated atria and ventricular tissue (either a moderator band, a trabeculae carnae, or papillary muscle) were rapidly excised and placed in a myograph for the determination of the following mechanical and electrical properties: 1) length-tension relations; 2) force-velocity relations; 3) spontaneous right atrial frequency of contraction; 4) refractory period; 5) force-frequency relations; 6) the response to paired electrical stimulation.

Sufficient data have not yet been accumulated to determine if differences

exists in the above parameters at the stages of development studied. However, it is apparent that the isolated muscle techniques will provide the data necessary to analyze the intrinsic mechanical properties of the heart as a function of age. Of particular interest was the observation that the heart of the fetus and term animal responded qualitatively and quantitatively like the adult to post-stimulation and post-extrasystolic potentiation. Recently, Arcilla and co-workers described an absence of post-extrasystolic potentiation of ventricular contraction in human newborns less than 2 weeks old. They did not believe that their observations could be accounted for by an increase in ventriculo-atrial regurgitation during cardiac catheterization and proposed instead, that an intrinsic difference existed in the mechanics of ventricular contraction between newborn infants and adults. Our findings fail to support such a view.

Proposed Course of Project: Sufficient data from additional animals in each age group are being accumulated to permit a statistical analysis of the results. In addition, the effects on isolated myocardium of digitalis glycosides and of altering the chemical environment by changing pH and gas tensions will be compared at different stages of development.

Honors and Awards: None

Publications: None

Serial No. - NHI-62 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Alterations in Regional Pulmonary Blood Flow in Patients with Congenital Heart Disease.

Previous Serial Number: NHI-40 (c)

Principal Investigator: William F. Friedman, M.D.

Other Investigators: Eugene Braunwald, M.D.
Andrew G. Morrow, M.D.

Cooperating Units: Clinic of Surgery, National Heart Institute

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: It has recently become possible to determine the distribution of pulmonary blood flow in an accurate and reproducible manner by scintillation scanning of the lungs after the infusion of ^{131}I-labeled macroaggregates of human albumin (^{131}I-MAA). In the present study lung scans were employed to investigate the patterns of regional pulmonary blood flow (PBF) associated with a variety of congenital cardiac malformations, to evaluate features of the bronchial circulation in selected patients with cyanotic heart disease, and to define the alterations in the patterns of pulmonary perfusion which follow operations such as superior vena cava-pulmonary arterial anastomosis and subclavian-pulmonary arterial anastomosis.

It is recognized that because of the influence of gravity on the low pressure pulmonary vascular bed, normal erect subjects have greater blood flow to the lung bases than to the apices. In patients with pulmonary venous hypertension, there is a relative increase in blood flow to the apices and a decrease in perfusion to the lung bases, and it has been shown that a linear relationship exists between the magnitude of the shift of blood flow towards the apices and the level of mean left atrial pressure. Investigations utilizing inhaled or injected radioactive gases have demonstrated that left-to-right circulatory shunts also increase blood flow to the apices and reduce the normal differences in perfusion between lung apex and base. Thus, the present investigation was also designed to study the distribution of PBF in patients with congenital heart disease in an effort to distinguish those with pulmonary arterial and venous hypertension from those with pulmonary arterial hypertension alone.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

The distribution of pulmonary arterial blood flow was evaluated by external scintillation scanning of intravenously administered ^{131}I-MAA in 61 normal subjects and in 100 patients with various congenital cardiovascular malformations. Intraaortic administration of ^{131}I-MAA demonstrated that blood flow through subclavian-pulmonary artery anastomoses is directed principally to the lung on the side of the anastomosis; the relative concentration of ^{131}I-MAA in each lung after intravenous injection provided an index of the patency of the anastomosis or of the development of pulmonary atresia or pulmonary hypertension. In contrast to the findings in patients with a patent subclavian-pulmonary shunt, scans obtained from patients with patent ductus arteriosus did not reveal a separation of the systemic arterial and systemic venous inflows to the lungs. The patency of superior vena caval-right pulmonary arterial anastomosis could be assessed after injection of ^{131}I-MAA into an upper-extremity vein.

Anomalies characterized by increased pulmonary blood flow and/or elevated pulmonary arterial pressures increased the ratio of pulmonary blood flow in the lung apices relative to that in the dependent lung zones. Anomalies characterized by elevated pulmonary venous pressure, such as cor triatriatum and mitral regurgitation, were readily detected by demonstrating both a decrease in blood flow to the lung bases as well as an increase to the apices. Thus, in patients with known pulmonary arterial hypertension (mean pressure >30 mm Hg) the ratio of upper/lower zone blood flow was always significantly higher if the arterial hypertension was accompanied by venous hypertension. For this reason, lung scans facilitated the screening of patients with pulmonary arterial hypertension for surgically correctable lesions such as cor triatriatum and mitral stenosis.

The method described is technically simple, without risk, easily applicable to large numbers of patients, and provides clinically important information concerning many forms of congenital heart disease.

Proposed Course of Project: Project completed.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Friedman, W. F., Braunwald, E., and Morrow, A. G.: Alterations in regional pulmonary blood flow in patients with congenital heart disease studied by readioisotope scanning. Circulation. In press.

197

Serial No. - NHI-63
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Sympathetic Innervation of the Developing Mammalian Heart: Biochemical and Histochemical Comparisons of Fetal, Neonatal, and Adult Myocardium.

Previous Serial Number: NIH-38

Principal Investigator: William F. Friedman, M.D.

Other Investigators: Peter E. Pool, M.D.
David Jacobowitz, Ph.D.
Shirley C. Seagren, B.A.
Eugene Braunwald, M.D.

Cooperating Units: Department of Pharmacology
University of Pennsylvania

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: Although the importance of the adrenergic nervous system in the control of cardiac contractility in the mature mammal has been well documented, its significance in the perinatal period is not clear. Physiologic and pharmacologic studies undertaken to assess the maturation of the autonomic control of the circulation have been largely concerned with the ability of young animals to respond to various physiologic stimuli such as hypoxemia and carotid sinus hypotension or to the injection of catecholamines. A number of observations have been made in rabbits of varying ages that suggest that the circulation of the newborn is under no, lesser, or comparable degrees of neural control, when compared to the adult. The temporal development of the separate factors comprising an integrated circulatory response, namely, the afferent, central, and efferent components of a vascular reflex, the responsiveness of the peripheral vasculature, and the direct inotropic and chronotropic effects on the myocardium have not yet been analyzed quantitatively.

The objective of the present investigation was to define more clearly the development of sympathetic innervation of the rabbit heart. Accordingly, the cardiac concentration of norepinephrine in fetal, neonatal and adult animals was employed as an index of the maturity of sympathetic innervation because the heart's stores of norepinephrine are localized almost exclusively in intracellular storage sites within the terminations of the sympathetic nerves. In

193

addition, the monoamine fluorescence technique of Falck was employed to evaluate histochemically the distribution of sympathetic nerves within the heart.

Rabbits were studied at term gestation, 2 days, 1, 2, 3, 5 and 12 weeks, and 1 year of age. The cardiac concentration of norepinephrine rose progressively from term gestation until adult levels were reached at approximately three weeks of age. Similar, small amounts of epinephrine were found in the hearts at all ages (<0.10 μg/gm). Substantially less change in the total concentration of adrenal catecholamines accompanied advancing age (0.51 ± 0.02 (S.E.) mg/gm at two days, 0.71 ± .08 at three and four weeks). At all of the ages studied the histochemical observations correlated well with the measured norepinephrine levels and at each stage of development the atria were noted to be more densely innervated than the ventricles.

It has been shown that the ordinary activity of the adrenergic nervous system may have minimal effects on the normal heart and that the intrinsic contractile state of the myocardium may not be influenced by alterations in endogenous catecholamine stores. However, it is clear that the force of contraction of the heart may be stimulated profoundly by an increase in the number of impulses traversing the sympathetic nerves whenever an imbalance exists between the cardiac output and the perfusion requirements of the peripheral tissues. When the latter situation occurs in the perinatal period, it would appear that the adrenal release of catecholamines may play a more critical compensatory role in maintaining ventricular contractility than in the adult.

Proposed Course of Project: The alterations in the normal development of cardiac autonomic innervation produced by pathologic states and by transplacental and postnatal administration of pharmacologic agents will be studied. Perhaps of greatest practical importance in this regard will be the determination of the influence of premature delivery and nutrition on subsequent autonomic development. The influence on the fetal heart of drugs, including reserpine and ganglionic blocking agents, commonly administered to hypertensive or toxemic mothers, will be studied, as will the effects on the fetal heart of the administration to the mother of thyroid hormone, and of thyroid ablation in the mothers.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Friedman, W. F., Pool, P. E., Jacobowitz, D., Seagren, S. C., and Braunwald, E.: Sympathetic innervation of the developing mammalian heart: Biochemical and histochemical comparisons of fetal, neonatal, and adult myocardium. Circulation Research. In press.

Serial No. - NHI-64
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Estimation of Left Ventricular Myocardial Function in Man by Means of Instantaneous Tension-Velocity-Length Relations

Previous Serial Number: NHI-25(c)

Principal Investigator: James H. Gault, M.D.

Other Investigators: John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.4
Professional:	.2
Others:	.2

Project Description: It has long been evident that standard hemodynamic measurements provide an incomplete description of the contractile properties of the left ventricle, and do not permit the quantitative comparison of myocardial contractile state among different patients. Recent observations in the isolated papillary muscle and in intact canine left ventricle indicate that a comprehensive description of myocardial contractile performance requires consideration of the relations between myocardial wall tension, fiber length, and the velocity of fiber shortening. In the present study the mechanics of left ventricular (LV) contraction in man have been analyzed by correlating LV dimensional changes during contraction, determined directly from high speed cineangiograms, with simultaneous high fidelity recordings of LV pressure. The mechanical characteristics of ventricular contraction have been expressed in a quantitative manner by deriving the extent and velocity of circumferential fiber shortening and LV wall tension (stress) throughout contraction.

These techniques were employed to characterize the mechanics of LV contraction in 6 patients without left heart disease, and in 9 patients with LV myocardial disease. In patients without LV disease wall tension reached a maximum level soon after the onset of ejection, then declined rapidly, while in patients with LV disease, tension fell only slightly during ejection. The extent of circumferential shortening, and the maximum fiber shortening rate, were consistently reduced in patients with LV disease at levels of wall tension comparable to those in patients without LV disease. The impairment of contractility in patients with LV disease was also evidenced by a similar reduction in fiber shortening rate at maximum tension, when velocity of fiber

shortening can be assumed to be equal to that of the contractile elements.

These observations indicate that LV myocardial function can be analyzed quantitatively in terms of the mechanical characteristics of ventricular contraction. Further, by focusing on the mechanical function of the ventricular muscle per se, these techniques permit the quantitative assessment of ventricular performance under altered loading conditions, such as with valvular and congenital cardiac lesions.

Proposed Course of Project: Project completed.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Gault, J. H., Ross, J., Jr., and Braunwald, E.: The contractile state of the left ventricle in man: instantaneous tension-velocity-length relations in patients with and without disease of the left ventricular myocardium. Circulation Research 22: 451, 1968.

Serial No.- NHI-65 (c)
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Left Ventricular Myocardial Function in Patients with Free Aortic Regurgitation, Determined by Instantaneous Tension-Velocity-Length Relations
Previous Serial Number: None
Principal Investigator: James.H. Gault, M.D.

Other Investigators: John Ross, Jr., M.D.
James W. Covell, M.D.

Cooperating Units: None

Man Years

Total:	.4
Professional:	.2
Others:	.2

Project Description: In patients with valvular cardiac lesions, it has not been possible to assess accurately the level of left ventricular (LV) myocardial performance by means of standard hemodynamic measurements. Recent studies in this laboratory have shown that the instantaneous relation between the velocity of fiber shortening (V_{CF}), determined directly from high-speed cineangiograms, and LV wall tension, calculated from ventricular radius and simultaneous high fidelity micromanometer recordings of LV pressure, provide a quantitative means of estimating LV contractility in patients without valvular disease. In the present study, these methods were employed to characterize the mechanics of LV contraction and myocardial contractile state, in ten patients with free aortic regurgitation.

Nine of the ten patients had severe cardiac symptoms, although in six, the cardiac index and LV end-diastolic pressure (LVEDP) were normal. In two of the latter patients, the maximum V_{CF} and V_{CF}-tension relations were normal [> 1.45 circumferences (circ)/sec. and >1.35 circ/sec at maximum tension (T_{max})]. In four patients in whom the cardiac index was reduced and the LVEDP elevated, the maximum V_{CF} and V_{CF}-T_{max} (maximum tension) relations were markedly depressed (average, 0.63 circ/sec and 0.38 circ/sec; in addition, maximum V_{CF} occurred early during ejection, when tension was relatively low, and V_{CF} then declined while tension continued to rise. In four patients with normal cardiac index and LVEDP, maximum V_{CF} and V_{CF}-T_{max} relations were slightly depressed (average 1.26 circ/sec and 1.10 circ/sec) despite increased diastolic fiber lengths; however, in these patients V_{CF} was maintained throughout contraction, reaching a peak level at or after T_{max}.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

It is concluded that characteristic alterations in the mechanics of LV contraction occur as the result of reduced myocardial performance in patients with severe aortic regurgitation, and that varying degrees of depression of LV contractility can be detected even when the cardiac index and LVEDP are normal.

Proposed Course of Project: Project completed, manuscript in preparation.

Honors and Awards: None

Publications: None

198

Serial No. - NHI-66

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Physiological Differences Between the Effects of Neuronally Released and Bloodborne Norepinephrine on Beta Adrenergic Receptors in the Arterial Bed of the Dog

Previous Serial Number: NHI-46

Principal Investigator: Gerald Glick, M.D.

Other Investigators: Stephen E. Epstein, M.D.
Andrew S. Wechsler, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:
Total:
Professional:
Other:

Project Description: This investigation was designed to define the role of the beta adrenergic receptors in the regulation of peripheral vascular resistance and to determine whether norepinephrine released from nerve terminals acts at the same receptor sites in the arterial bed as injected norepinephrine. In 34 anesthetized dogs the skinned hindlimb was perfused at a constant flow rate, and in 8 dogs a segment of the splanchnic vascular bed was similarly perfused through the aorta. The hemodynamic responses of the isolated perfused beds of control dogs were compared with those of dogs in which the alpha receptors had previously been blocked with phenoxybenzamine, thereby maximizing any contribution of the beta receptors. In the control animals, both carotid sinus hypotension and norepinephrine administered directly into the perfused segment increased vascular resistance. In those animals subjected to alpha-receptor blockade, carotid sinus hypotension still caused reflex vasoconstriction, though it was considerably attenuated, but intra-arterially injected norepinephrine produced vasodilation. Following subsequent beta-receptor blockade, no potentiation of reflex constriction occurred, although the response to injected norepinephrine reverted to constriction. These findings suggest that norepinephrine released from nerve terminals in the arterial tree does not produce physiologically significant beta-receptor stimulation; humorally transported norepinephrine, however, stimulates both alpha and beta receptors.

Proposed Course of Project: Project completed.
Honors and Awards: None
Publications: ARTICLE PUBLISHED IN A PERIODICAL

Glick, G., Epstein, S. E., Wechsler, A. S., and Braunwald, E.: Physiological differences between the effects of neuronally released and bloodborne norepinephrine on beta adrenergic receptors in the arterial bed of the dog. Circulation Res. 21: 217-227, 1967.

Serial No. - NHI-67
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Relative Importance of the Carotid and Aortic Baroreceptors in the Reflex Control of Heart Rate

Previous Serial Number: None

Principal Investigator: Gerald Glick, M.D.

Other Investigators: James W. Covell, M.D.

Cooperating Units: None

Man Years

Total:
Professional:
Other:

Project Description: In nine dogs the cerebral circulation was isolated from the rest of the arterial bed and was perfused by a donor dog. Changes in perfusion pressure could then be produced independently in the carotid sinus and aortic arch areas by injection of vasoactive drugs. In four dogs stimulation of the aortic arch receptors produced greater effects on heart rate than did sinus stimulation. In the remaining five animals, the effects on heart rate of stimulating the two baroreceptor areas were similar. The carotid sinus never appeared more important. In seven studies, perfusion pressures were altered simultaneously in opposite directions. In each case, heart rate slowed as a result of the stimulation caused by increased pressure in one receptor region, despite the concomitant withdrawal of baroreceptor stimulation in the other area. Thus, both baroreceptor areas are important in the control of heart rate, although the aortic arch areas sometimes predominate. Moreover, a positive input produced by raising pressure is a more powerful stimulus than the negative input of lowering perfusion pressure.

Proposed Course of Project: Project completed.

Honors and Awards: None

Publications: ARTICLE PUBLISHED IN A PERIODICAL

Glick, G., and Covell, J. W.: Relative importance of the carotid and aortic baroreceptors in the reflex control of heart rate. Am. J. Physiol. In press.

500

Serial No.- NHI-68
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Glucagon: Its Enhancement of Cardiac Performance in the Cat and Dog, with Special Reference to Persistence of Its Inotropic Action Despite Beta-Receptor Blockade with Propranolol

Previous Serial No.: None

Principal Investigator: Gerald Glick, M.D.

Other Investigators: William W. Parmley, M.D.
Andrew S. Wechsler, M.D.
Edmund H. Sonnenblick, M.D.

Cooperating Units: None

Man Years
Total: .8
Professional: .4
Other: .4

Project Description: Catecholamines may exert their positive inotropic and chronotropic actions by activating the enzyme adenyl cyclase, which catalyzes the conversion of ATP to adenosine 3', 5'-monophosphate (cyclic AMP). Furthermore, glucagon is known to increase formation of cyclic AMP in many isolated tissues. The relevance of these observations to the heart was studied in 21 isolated cat papillary muscle preparations, in 13 spontaneously beating cat atria, in 15 intact dog hearts and in 4 isolated perfused dog hindlimbs. In each papillary muscle preparation, addition of glucagon produced marked increases in maximal developed tension, averaging 36 $\pm$ 4.2% (SEM) ($p < 0.01$), and shifted the force velocity curve upwards and to the right, indicating augmented contractility. Glucagon always increased the rate of the spontaneously beating atrium, the rise averaging 28.8 $\pm$ 5.5 contractions/min ($p < 0.01$).

In the dog, myocardial performance was markedly augmented by the i.v. administration of 50 µg/Kg glucagon as indicated by an average increase of 72.2 $\pm$ 18.4% ($p < 0.01$) in left ventricular peak dp/dt and of 58.9 $\pm$ 12.8% ($p < 0.01$) in force recorded by a strain gauge arch, despite an average decrease of 3.8 $\pm$ 1.2 cm H_2O ($p < 0.02$) in left ventricular end-diastolic pressure. Heart rate rose an average of 38.7 $\pm$ 10.9 beats/min ($p < 0.02$). Small but significant decreases in peripheral vascular resistance were produced. Single i.v. injections produced effects lasting 15-20 minutes. Propranolol did not prevent the inotropic responses in

either the cat or dog preparations but markedly decreased the chronotropic effects. Thus, glucagon is a powerful inotropic and chronotropic agent.

Proposed Course of Project: Project completed.

Honors and Awards: None

Publications

ARTICLE PUBLISHED IN A PERIODICAL:

Glick, G., Parmley, W. W., Wechsler, A. S., and Sonnenblick, E. H. Glucagon: Its enhancement of cardiac performance in the cat and dog, with special reference to persistence of its inotropic action despite beta-receptor blockade with propranolol. Circulation Res. In press.

207

Serial No. - NHI-69
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Role of Pulmonary Stretch Receptors in the Control of Cardiovascular Function

Previous Serial Number: None

Principal Investigator: Gerald Glick, M.D.

Other Investigators: Andrew S. Wechsler, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Man Years

Total:	.4
Professional:	.2
Other:	.2

Project Description: Although it is recognized that stimulation of stretch receptors in the lungs produces marked reflex alterations in respiration, it has generally been assumed that these receptors do not influence the autonomic nervous control of the cardiovascular system. To investigate this problem, experiments were conducted on six dogs on total cardiopulmonary bypass and on four dogs in which a hindlimb was perfused at a constant flow rate with a sigmamotor pump. In all preparations, heart rate was monitored and changes in peripheral vascular resistance were assessed by recording perfusion pressure in the aorta in the animals on total bypass and in the limb of the animals with the perfused hindlimb; changes in perfusion pressure reflected alterations in resistance. Myocardial contractile force was monitored with a ventricular strain gauge arch.

When the lungs were suddenly expanded by a positive pressure of 20 mm. Hg, marked vasodilation occurred in every instance, the fall in systemic and limb perfusion pressures averaging 18 $\pm$ 5.8% (SEM) (avg. 16 $\pm$ 5.2 mm Hg) and 32.7 $\pm$ 7.7% (avg. 26 $\pm$ 8.7 mm Hg) respectively, heart rate generally slowed, and myocardial contractile force decreased. By varying the amount of distending pressure from 5 to 20 mm Hg, stepwise alterations in the extent of vasodilation could be produced. After vagotomy, the cardiovascular response to sudden lung inflation was either abolished or markedly attenuated. Thus, this reflex links the pulmonary to the cardiovascular system; the pulmonary receptors are sensitive to stretch, and the afferent pathway runs predominantly in the vagus nerves.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Proposed Course of Project Project completed.

Honors and Awards: None

Publications: None.

Serial No. - NHI-70
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Influence of Thyroid State on the Intrinsic Contractile Properties of Skeletal Muscle

Previous Serial Number: None

Principal Investigator: Herman K. Gold, M.D.

Other Investigators: James F. Spann, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: Although prolongation of the contractile process of skeletal muscle in myxedema has been documented in man, the effects of hyper- and hypothyroidism on the intrinsic function of isolated skeletal muscle are not clear. Accordingly, contractile properties of soleus muscles of euthyroid, hyperthyroid and hypothyroid rats were studied in a myograph. Tetanic contractions at a stimulation frequency of 100/sec were examined.

Maximum isometric tension was essentially identical in muscles from hyperthyroid and euthyroid animals, but was significantly depressed in muscles from hypothyroid rats. The rate of tension development was increased in hyperthyroid and decreased in the hypothyroid muscles. The duration of active state was also altered by changes in thyroid function; it was shortened in hyperthyroidism and prolonged in hypothyroidism. The mean rate of relaxation was increased above the normal value in hyperthyroid muscles and slowed in the hypothyroid muscles. Thus, it is apparent that hyperthyroidism and myxedema result in profound alterations in the intrinsic contractile properties of skeletal muscle.

These data from isolated muscle can be utilized to formulate several concepts related to the abnormalities of muscle function in vivo in these conditions. In hypothyroidism, tension is reduced as a consequence of the lowered intensity of the active state. The slow rate of relaxation in hypothyroidism and the rapid rate of relaxation in hyperthyroidism are intrinsic properties of these muscles. The weakness observed in vivo in hyperthyroidism may be due to a lack of complete fusion of contractile events due to the reduction

205

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

of the duration of active state in the absence of an increase in the rate of motor nerve impulses in this disorder. Furthermore, such a lack of fusion would result in constantly varying total tension and thus produce a tremor such as is frequently seen in hyperthyroidism.

Proposed Course of Project: To be completed.

Honors and Awards: None

Publications: None

Serial No.- NHI-71
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1967

Project Title: Contractile Characteristics of Cardiac Muscle from Animals Receiving Excess Growth Hormone

Previous Serial Number: None

Principal Investigator: Herman K. Gold, M.D.

Other Investigators: James F. Spann, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years (computed for the 12 month period)

Total:	.4
Professional:	.2
Other:	.2

Project Description: Excessive growth hormone in humans, the acromegalic state, is associated with enlargements of the viscera, including the heart, and is thought to be associated with abnormal cardiac function. However, it is not known whether the hypersomatropic state is associated with primary abnormalities of cardiac muscle function. Accordingly, cats have been given excessive doses of purified bovine growth hormone for three weeks and the contractile properties of right ventricular papillary muscle from these hearts has been studied in a myograph. The preliminary results from 3 such animals showed no change in heart size and slightly abnormal function of the cardiac muscle. The rate of rise of isometric tension and the maximum isometric tension developed were reduced in muscles from these cats. There were no definitive changes in the velocity of isometric shortening. The response of these muscles to digitalis glycosides appears to be greater than the response in normal muscle. In two of the three animals studied there was evidence of hyperthyroidism, probably due to contamination of the growth hormone by thyroid stimulating hormone, a factor which could obscure depression of function in the isolated heart muscle.

Proposed Course of Project: The above studies will be extended to increase the number of animals studied. Growth hormone will be given for a longer period of time before the cardiac muscle function is defined. The effect of thyroid stimulating hormone will be prevented by destroying the thyroid land with I^{131} and maintaining the animals on constant doses of thyroxine.

Honors and Awards: None

Publications: None

Serial No. - NHI-72
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Control of Myocardial Oxygen Consumption: Relative Importance of Contractility and Tension Development

Previous Serial Number: NIH-49

Principal Investigator: Thomas P. Graham, Jr., M.D.

Other Investigators: James W. Covell, M.D.
John Ross, Jr., M.D.
Edmund H. Sonnenblick, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: The purpose of this study was to provide a direct comparison of the quantitative effects of tension development and myocardial contractile state on the heart's oxygen consumption ($M\dot{V}O_2$). This was accomplished by varying peak developed tension (PDT) at a constant level of contractility and conversely by varying contractility at a constant PDT in the same animal.

An isovolumically contracting dog left ventricular preparation was utilized with total cardiopulmonary bypass and retrograde perfusion of the systemic circulation. Left ventricular volume was varied by injecting or withdrawing known amounts of saline into an intraventricular balloon. Left ventricular pressure (LVP), the rate of left ventricular pressure development (LV dp/dt), aortic pressure, the electrocardiogram, A-$\dot{V}O_2$ difference, and estimated wall tension were monitored. The experimental protocol was to measure $M\dot{V}O_2$ at varying levels of PDT produced by varying left ventricular volume. Norepinephrine was then infused into the ascending aorta at rates ranging from 0.76 to 7.6 μg/min in order to increase myocardial contractile state. Left ventricular volume was then varied in order to match the level of PDT achieved in each of the pre-norepinephrine states. Analysis of data was facilitated by use of a digital computer by which force and velocity parameters were calculated for representative beats. The contractile element velocity extrapolated to zero load (V_{max}) was used as the index of contractile state.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

$M\dot{V}O_2$ consistently increased with changes in both PDT and contractile state. Multiple regression analysis yielded the following equation: $M\dot{V}O_2$ (μl/beat/100 g LV) = -35 + .25 PDT (g/cm^2) + 1.43 V_{max} (cm/sec). It is concluded that the oxygen cost of alterations in contractile state (V_{max}) is substantial and of an order of magnitude similar to that associated with alterations in myocardial wall tension.

Proposed Course of Project: Project completed.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Graham, T. P., Jr., Covell, J. W., Sonnenblick, E. H., Ross, J., Jr., and Braunwald, E.: Control of myocardial oxygen consumption: Relative influence of contractile state and tension development. J. Clin. Invest. 47: 375-385, 1968

Serial No. - NHI-73
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of Angiographic Dye on Isometric Contraction and Force-Velocity Characteristics of Cat Papillary Muscle

Previous Serial Number: None

Principal Investigator: Dean T. Mason, M.D.

Other Investigators: James F. Spann, Jr., M.D.
G. David Beiser, M.D.
Herman K. Gold, M.D.

Cooperating Units: None

Man Years (computed for the 12 month period)

Total:	.8
Professional:	.4
Other:	.4

Project Description: Although angiographic contrast media produce marked changes in circulatory dynamics, their effects on the contractile state of the myocardium have not been completely defined. Thus, the direct influence of 10 to 50% concentrations of Hypaque-75% was studied on both isotonic and isometric contractions of 8 isolated right ventricular cat papillary muscles. Hypaque 10% reduced the force-velocity relations, contractile element velocity at a constant load of 0.5 Gm/mm^2 (V_{CE}) declining from 0.94 to 0.80 muscle lengths/sec (-15%). V_{CE} was diminished strikingly (-25%) with 20% and (-50%) with 50% Hypaque. Peak isometric tension fell markedly from 5.7 to 1.6 Gm/mm^2 (-72%) and the maximal tension during paired electrical stimulation was reduced from 9.2 to 1.7 Gm/mm^2 (-82%) with 50% Hypaque. The rate of tension development was diminished, 23.7 to 6.0 Gm/mm^2/sec (-75%), while the time to peak tension was not altered by 50% Hypaque. The actions of Hypaque were quantitatively similar in 2 cats with experimentally produced right ventricular failure. Following removal of contrast material from the muscle bath, V_{CE} was augmented 11% in 6 cats, perhaps reflecting an increase in contractile state due to residual calcium from the Hypaque solution. It is concluded that angiographic dye, even in small concentrations, exerts a profound direct negative inotropic action on heart muscle which appears to be largely responsible for the transient hypotension observed clinically; the increase in contractility might contribute, in part, to the sustained phase of hypertension and elevated cardiac output.

210

Serial No. - NHI-73

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Proposed Course of Project: Project nearly completed. The contractile effects of hyperosmolarity per se are being studied utilizing Sucrose. In addition, the contractile effects of calcium-free Hypaque are under investigation.

Honors and Awards: None

Publications: None

211

Serial No. - NHI-74
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Alterations of Left Ventricular Performance and Myocardial Mechanics in Patent Ductus Arteriosus and Ventricular Septal Defect.

Previous Serial Number: None

Principal Investigator: Dean T. Mason, M.D.

Other Investigators: Robert Zelis, M.D.
James F. Spann, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: With equal left-to-right shunts cardiocirculatory effects of patent ductus arteriosus (PDA) and ventricular septal defect (VSD) are generally considered to be similar. The effects of acute, controlled, and metered PDA and VSD were studied in 7 dogs by use of external shunts, total stroke volume (SV) being increased from an average of 14.7 to 28.7 cc. and a 2 to 1 pulmonary to systemic flow ratio with both lesions. With PDA, left ventricular (LV) end-diastolic pressure and volume (EDV) rose by 5 mm.Hg and 19 cc.; with VSD these variables rose less (1 mm. Hg and 5 cc., $< .01$). Although contractility remained unchanged, mean systolic ejection rate of 77 cc./sec. rose by 79% with PDA and by 116% with VSD, the ejection fraction (total SV/EDV) increased from 0.44 to 0.57 with PDA and to 0.76 with VSD. With both PDA and VSD, tension decline during ejection was accelerated above control because of more rapid diminution of LV size during systole. Consequently, contractile element (CE) velocity was increased, as were CE work and CE power, to a greater extent with PDA than VSD. Although the decline in instantaneous impedance to ejection allowed LV muscle to shorten faster and further, the associated increase in external energy output and CE work was greater in PDA than VSD since the LV must eject the entire SV into the high pressure aorta, while in VSD a large portion of this volume is shunted directly into the low pressure right ventricle. It is concluded that LV performance is compromised to a greater extent by a PDA than a VSD, with equal shunts, and that this difference is explicable by a consideration of myocardial mechanics.

Proposed Course of Project: Project completed.

Honors and Awards: None

Publications: In preparation.

Serial No.- NHI-75
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Assessment of Myocardial Contractility in Man: Relationship between the Rate of Pressure Rise and Developed Pressure Throughout Isometric Left Ventricular Contraction

Previous Serial No.: None

Principal Investigator: Dean T. Mason, M.D.

Other Investigators: Edmund H. Sonnenblick, M.D.
James W. Covell, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: The maximum rate of intraventricular pressure rise (peak dp/dt) has been used extensively in assessing myocardial contractility. However, this variable also is affected by alterations in ventricular afterload through opening of the semilunar valves. It was considered that the relationship between dp/dt and developed pressure $\frac{(dp/dt)}{P}$ throughout isovolumetric contraction might afford a more accurate measure of contractility. In six cat papillary muscles contracting isometrically, from any given preload $\frac{dp/dt}{P}$ always varied directly with the contractile state; $\frac{dp/dt}{P}$ rose slightly as preload was increased, but this relationship was not affected by changes in afterload. In ten experiments in an intact canine heart preparation in which ventricular end-diastolic pressure was constant, $\frac{dp/dt}{P}$ again was directly related to contractility, as reflected in velocity of shortening of unloaded fibers (V_{max}), either when contractility was enhanced (norepinephrine, calcium, acetylstrophanthidin) or depressed (pentobarbital).

High-fidelity left ventricular pressure and dp/dt were recorded in conscious patients and $\frac{dp/dt}{P}$ determined throughout isovolumic contraction. In ten patients isoproterenol shifted the relation, raising $\frac{dp/dt}{P}$ at maximum isometric pressure by an average of 59%, and muscular exercise increased it by 52%. Interventions known not to influence the contractile state, such as

213

elevation of end-diastolic pressure by leg-raising, resulted in only minor changes in $\frac{dp/dt}{P}$. In conclusion, the determination of $\frac{dp/dt}{P}$ throughout isovolumic contraction provides a useful, simple and experimentally valid approach to the assessment of the contractile state of the heart in intact man.

Proposed Course of Project: Project completed

Awards and Awards: None

Publications: In preparation

Serial No.- NHI-76 (c)
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Clinical Applications of the Radioisotope-Angiocardiogram for the Rapid Sequential Visualization of the Heart and Great Vessels in Man: Use of a Gamma-Scintillation Camera Following the Selective Injection of Technetium-^{99m}.

Previous Serial Number: None

Principal Investigator: Dean T. Mason, M.D.

Other Investigators: Eugene Braunwald, M.D.
William Ashburn, M.D.
John Harbert, M.D.

Cooperating Units: Department of Nuclear Medicine, NIH

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: The recent development of the instantaneously sensing gamma-scintillation camera and the rapidly scanning television tube, capable of recording wide-field images over the precordium produced by trace concentrations of gamma-emitting radioisotopies at 1/60 sec. intervals as it flows through the circulation, and the storage of the radioisotope picture on video magnetic tape, has provided a means for visualizing the anatomic features of the heart and great vessels without the hazards involved in introducing a radiopaque agent. Sodium pertechnetate ($^{99m}TcO_4^-$) produces no hemodynamic effects or untoward actions and is quickly excreted, with an effective half-life of 6 hours. Following injection, the rapidly changing distribution of radioactivity within the heart is registered by the scintillation-television system which includes no inherent dead space or overlap of the scanned field from frame to frame. The tape replay is available immediately and sequential integrated pictures are possible at any rate up to 60/sec. Radioisotope images of longer intervals are easily obtained by the integration of successive stop-action fields reproduced on a high resolution television monitor and photographed on rapidly developed film. In addition, the replay may be gated and tracked, allowing selected phases to be integrated and time-concentration curves of blood flow.

Five to 12 millicuries of $^{99m}TcO_4^-$ in 2 to 8 cc. was rapidly injected

into selected cardiac chambers at the time of diagnostic catheterization in 50 patients with a variety of congenital and acquired forms of heart disease. Movement of radioisotope closely reflected the hemodynamic alterations caused by these conditions, as determined by contrast angiocardiography prior to the administration of $^{99m}TcO_4^-$. The radioisotope-angiocardiogram provides a new approach for the visualization of the cardiovascular system, does not require the use of radiopaque media, is safer and does not disturb circulatory function.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: In preparation.

Serial No.- NHI-77 (c)
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Diagnostic Value of the Configuration of the Transaortic Pressure Gradient for the Differentiation between Idiopathic Hypertrophic Subaortic Stenosis and the Discrete Forms of Left Ventricular Outflow Obstruction

Previous Serial Number: None

Principal Investigator: Dean T. Mason, M.D.

Other Investigators: Lawrence Sorel Cohen, M.D.
James F. Spann, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years

Total:	.8
Professional:	.4
Others:	.4

Project Description: Since the obstruction in idiopathic hypertrophic subaortic stenosis (IHSS) is dynamic, whereas that in discrete aortic stenosis (AS) is fixed, it was considered that the shape of the transaortic pressure gradient might be altered in IHSS and thereby provide a means of differentiating these two conditions. In 22 patients with IHSS the peak of the arterial pulse always occurred in the initial half of the systolic ejection period, while in 37 of 40 patients with AS it appeared in the final half. Peak left ventricular pressure was delayed significantly ($p < 0.01$) in IHSS compared to AS; the interval from onset of ejection to peak pressure divided by the ejection period was 0.59 ± 0.01 in IHSS and 0.49 ± 0.01 in AS. It is suggested that the early arterial and late ventricular peaks in IHSS are related to absence of obstruction to ejection early in systole. Most importantly, the ratio of the mean pressure gradient during the first half of ejection to that of the last half averaged 0.59 ± 0.04 in IHSS. In contrast, this ratio averaged 1.24 ± 0.06 in 23 patients with valvular aortic stenosis, 1.13 ± 0.06 in 12 patients with discrete subvalvular stenosis, and 1.85 ± 0.09 in 5 patients with supravalvular stenosis. This ratio allowed complete separation of IHSS from AS; a ratio < 1.00 indicated IHSS while a ratio of < 1.00 indicated fixed obstruction. Whether the brachial arterial pulse, appropriately adjusted for time, or the central aortic pulse was used did not substantially alter these results. Thus, analysis of the configuration

217

of the transaortic pressure gradient affords a reliable separation of AS from IHSS, is of even greater diagnostic value than previous descriptions of hemodynamic events, and does not require a provocative maneuver.

Proposed Course of Project: Project completed

Honors and Awards: None

Publications: In preparation

Serial No.- NHI-78 (c)

1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanics of Left Ventricular Contraction in Mitral Regurgitation

Previous Serial Number: None

Principal Investigator: Charles B. Mullins, M.D.

Other Investigators: James H. Gault, M.D.
Kevin O'Brien, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: Determination of instantaneous force velocity relations throughout the cardiac cycle has provided quantitative analysis of left ventricular function. Previous studies in this laboratory have revealed that myocardial function can be estimated in the presence of aortic insufficiency by force-velocity analysis of left ventricular function. This study proposes to evaluate the force-velocity relation in the left ventricle in patients with mitral insufficiency to delineate the effects of early unloading.

Accordingly, left ventricular cineangiograms were performed in the right anterior oblique position to outline the left ventricular chamber while monitoring left ventricular pressure in patients with significant mitral regurgitation. Measurements were then taken from the cine films, frame by frame, in the minor axis of the left ventricle throughout a cardiac cycle and matched with simultaneous pressure recordings. Wall tension and velocity of shortening was calculated in the minor left ventricular circumference every 17 msec. The force-velocity relations could then be determined by plotting the wall tension against the velocity of shortening throughout the cardiac cycle and compared with similar studies in patients with normal left ventricles. Eight patients with mitral regurgitation showed normal force-velocity relations as opposed to the previous findings in patients with aortic regurgitation which were strikingly abnormal. This suggests that myocardial function is normal in these patients with mitral regurgitation.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Proposed Course of Project: Patients with mitral regurgitation and with mixed mitral regurgitation and myocardial disease which are diagnostically studied on the unit during the remainder of the year will be analyzed to further this study. The paper will be submitted when the study is complete.

Honors and Awards: None

Publications: None

Serial No.- NHI-79
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effect of End-Diastolic Volume on the Velocity of Left Ventricular Circumferential Fiber Shortening

Previous Serial Number: None

Principal Investigator: Kevin P. O'Brien, M.D.

Other Investigators: John Ross, Jr., M.D.
James H. Gault, M.D.
James W. Covell, M.D.

Cooperating Units: None

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: When contractile state is constant, the two principal determinants of the velocity of myocardial fiber shortening are myocardial wall tension (afterload) and end-diastolic volume (end-diastolic length). Under controlled conditions, velocity of circumferential fiber shortening (V_{CF}), is inversely related to wall tension and directly related to instantaneous fiber length, but the effects of varying end-diastolic volume on V_{CF} and tension in normal intact closed-chest dogs has not been examined when ventricular geometry is known. Five closed-chest dogs were studied. The EDV was varied to several levels in each dog by either infusing or withdrawing blood from the animal. At each level of EDV, high speed biplane cineangiograms were recorded after injection of contrast material into the left ventricle. Measurements of left ventricular dimensional changes during contraction were made and correlated with simultaneous high fidelity recordings of left ventricular pressure. The extent and velocity of circumferential fiber shortening, together with the time course of left ventricular wall tension were calculated, and instantaneous relations between tension, velocity and length were examined. End-systolic and end-diastolic ventricular volumes were calculated from the biplane cineangiograms. Preliminary observations indicate that calculated left ventricular wall tension is significantly higher throughout beats beginning at higher end-diastolic volumes. V_{CF} is inversely proportional to left ventricular wall tension, and this effect seems to counteract the expected influence of fiber length in increasing V_{CF}. Therefore, the data suggest that V_{CF} may be unchanged or reduced under these conditions.

284

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Proposed Course of Project: This study is still in the process of analysis.

Honors and Awards: None

Publications: None

222

Serial No. - NHI-80 (c)

1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Cardiovascular Effects of Glucagon in Man

Previous Serial Number: None

Principal Investigator: William W. Parmley, M.D.

Other Investigators: Gerald Glick, M.D.
Edmund H. Sonnenblick, M.D.

Cooperating Units: Cardiology Department, Peter Bent Brigham Hospital, Boston, Massachusetts

Man Years

Total:	.4
Professional:	.2
Other:	.2

Project Description: Recent studies in animals have demonstrated that glucagon has substantial positive inotropic and chronotropic effects in the presence of beta-adrenergic receptor blockade, catecholamine depletion, or full digitalization. Accordingly, the present study was undertaken to evaluate the cardiovascular effects of glucagon administration in man during the course of routine diagnostic cardiac catheterization. In 11 patients glucagon, 3 to 5 mg. i.v., resulted in significant increases in cardiac index, mean arterial pressure, heart rate, and the maximum rate of left ventricular pressure development, with no significant change in left ventricular end-diastolic pressure, or systemic vascular resistance. Onset of action after a single dose was evident in 1 to 3 minutes, reached a maximum in 5 to 7 minutes and lasted 10 to 15 minutes. In 6 patients receiving only 1.0 mg. glucagon i.v., the only significant changes were an increase in cardiac index and a decrease in systemic vascular resistance.

It is concluded that glucagon has a beneficial effect on cardiac performance in man, and with its demonstrated action in the absence of catecholamines and in the presence of full digitalization, it would appear to be a useful cardiotonic drug for the therapy of acute heart failure.

Proposed course of project: Project completed

Honors and awards: None.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

ARTICLE PUBLISHED IN A PERIODICAL:

Parmley, W. W., Glick, G., and Sonnenblick, E. H.: The cardiovascular effects of glucagon in man. Submitted for publication.

224

Serial No. - NHI-81
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Integrity of Energy Stores in Cat Papillary Muscle; Effect of Changes in Temperature and Frequency of Contraction on High Energy Phosphate Stores

Previous Serial Number: None

Principal Investigator: Peter E. Pool, M.D.

Other Investigators: Brian M. Chandler, M.D.
Edmund H. Sonnenblick, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: Although isolated cat papillary muscles have proved useful in a variety of physiologic and pharmacologic studies, the energetic integrity of this preparation has been questioned. In order to study this problem, papillary muscles (avg. diam - 2.3 mm) were equilibrated in oxygenated Krebs solution for one hour at 26°C at rest or contracting at various frequencies and temperatures and for various durations. In addition, right ventricular biopsies were obtained from cats in vivo. Specimens were frozen in liquid isopentane for determination of ATP and creatine phosphate (CP). In vivo total energy stores (Σ CP + ATP) were 12.0 $\pm$ 0.8 μmoles/g. In papillary muscles at rest, CP + ATP was 16.7 $\pm$ 0.6 μmoles/g, significantly higher than in vivo ($p < .01$). In vitro at 12/min, ΣCP + ATP was 16.0 $\pm$ 1.0 μmoles/g, not different from resting papillary muscles but higher than in vivo. At 30 or 60/min, Σ CP + ATP was reduced to 13.7 $\pm$ 1.1 and 11.1 $\pm$ 0.7 μmoles/g ($p < .05$). Neither increasing in vitro temperature from 26°C to 37°C nor extending the duration of stimulation from 1 to 3 hours at 12/min significantly altered Σ CP + ATP. It is concluded that papillary muscles even of moderately large diameter are energetically intact for at least 3 hours at frequencies of 12/min at 26°C. Further, energy stores are even greater than those found in vivo. At rates of 30 or 60/min energy stores may be limited.

Proposed Course of Project: Project completed.
Honors or Awards: None

225

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Publications:

Pool, P. E., Chandler, B. M., Sonnenblick, E. H., and Braunwald, E.: Integrity of energy stores in cat papillary muscle; effect of changes in temperature and frequency of contraction on high energy phosphate stores. Circulation Res. 22: 213-219, 1968.

Serial No.- NHI-82
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Mechanochemistry of Cardiac Muscle

Previous Serial Number: NHI-41

Principal Investigator: Peter E. Pool, M.D.

Other Investigators: Edmund H. Sonnenblick, M.D.
Brian M. Chandler, M.D.

Cooperating Units: None

Man Years (computed for the 12 month period)

Total:	.4
Professional:	.2
Other:	.2

Project Description: The purpose of this study was to define the chemical energetics of cardiac muscle by measuring the utilization of ATP and creatine phosphate (CP) in the papillary muscle of cat heart. To accomplish this, cat right ventricular papillary muscles were metabolically inhibited with iodoacetic acid and nitrogen to prevent further production of ATP and CP. The basal consumption of high energy phosphates was determined in resting muscles without tension by freezing muscles at various times after metabolic inhibition and assaying these muscles for CP and ATP. It was found that the utilization of high energy phosphates could be accounted for by the disappearance of CP and ATP.

The basal rate of CP and ATP consumption was 0.77 μmoles/g/min. Increasing resting tension (or muscle length) was found to increase basal high energy phosphate consumption in a linear fashion over the physiologic range of muscle lengths.

In addition, CP and ATP utilization was determined in isometrically contracting muscles. They were stimulated to contract 10-50 times at the top of their previously determined length-tension curves. The utilization of CP and ATP by these muscles could be accounted for by a prediction equation with terms for the number of activations and the calculated contractile element work. The mechanochemical efficiency of cardiac muscle, defined as the work performed divided by the energy cost of work plus activation, was 33% in these studies, a value similar to that of skeletal muscle previously determined by others.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Other metabolically inhibited muscles were stimulated to contract isotonically with varying afterloads to determine the relation of external work and fiber shortening to energy utilization in cardiac muscle. The efficiency of energy utilization for the performance of internal contractile element work was 0.0067 μmoles ~P/g-cm of work and for external work was 0.0031 μmoles ~P/g-cm (~P = CP + ATP). The findings provided a demonstration of the Fenn effect in cardiac muscle and explain the well known discrepancy in energy cost when cardiac work is increased by increasing pressure load as opposed to increasing volume load.

Further studies were carried out to determine whether the increase in myocardial contractility associated with the effects of norepinephrine was associated with an increase in energy utilization proportionate to the increase in work performance or whether a disproportionate increase in energy utilization associated with an increase in contractile state was present. Norepinephrine-treated papillary muscles used 115% as much ~P as control muscles in only 59% as many contractions and while performing only 87% as much work. It was concluded that the "oxygen wasting" effect of NE on heart muscle may result from the increased utilization of energy associated with an increased contractile state.

Finally, a study was designed to determine whether a defect in energy utilization could be the fundamental defect in the congestive heart failure state. The relation of energy utilization to tension development was studied in metabolically inhibited papillary muscles obtained from normal cats and cats with experimental right ventricular failure secondary to pulmonary artery constriction. While the average muscle from cats with heart failure performed 13% as much work, although activated 64% as many times, the average amount of energy used was only 7% of that used by normal muscles. It was concluded that in heart failure the net utilization of ~P is reduced, but only in relation to the reduction in contractile element work, and that the direct conversion of chemical energy to mechanical work is not an inefficient process in this state.

Proposed Course of Project: Project completed.

Honors and Awards: None

ARTICLES PUBLISHED IN A PERIODICAL:

Pool, P. E., and Sonnenblick, E. H.: The mechanochemistry of cardiac muscle. I. The isometric contraction. J. Gen. Physiol. 50: 951-965, 1967.

Pool, P. E., Chandler, B. M., and Sonnenblick, E. H.: The mechanochemistry of cardiac muscle. II. The isotonic contraction. Circulation Res. 22: April, 1968.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Chandler, B. M., Sonnenblick, E. H., and Pool, P. E.: The mechanochemistry of cardiac muscle. III. Effects of norepinephrine on the utilization of high energy phosphates. Circulation Res. 22: June, 1968.

Serial No.- NHI-83
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Relation of Myofibrillar ATPase Activity to Alterations in Contractile State of the Heart

Previous Serial Number: None

Principal Investigator: Shirley Seagren

Other Investigators: Peter E. Pool, M.D.

Cooperating Units: None

Man Years (computed for the 12 month period)

Total:	.6
Professional:	.3
Other:	.3

Project Description: In experimental heart failure, myofibrillar ATPase activity has been found to be depressed. This depression has been correlated with reduced contractility of the associated right ventricular papillary muscles. It has been proposed that myofibrillar ATPase activity may be directly related to alterations in the contractile state of the heart. In order to test this hypothesis, drugs known to increase the contractile state of the heart were added to myofibrillar preparations. In addition to these in vitro interventions, contractility was altered in vivo by inducing hyperthyroidism. ATPase activity was then assessed in myofibrillar preparations made from the hearts of these animals.

After isolation of myofibrils from the left ventricle, ATPase activity was measured in μmoles Pi released per mg of protein per minute. Determinations were performed at 37°C in the presence of 5 mM ATP and 5 mM $MgCl_2$ at pH 7.0. The addition of 5 mM sodium azide to the incubating medium eliminated mitochondrial ATPase contamination. The in vitro addition of 10^{-6}M ouabain produced no significant change in ATPase activity while addition of 10^{-6}M NE depressed activity somewhat from a normal value of .21 ± .01 to .17 ± .02 μmoles Pi/mg/min. Myofibrillar ATPase activity in the hyperthyroid state was lowered from .19 ± .01 to .16 ± .01 μmoles Pi/mg/min. These preliminary results suggest that myofibrillar ATPase activity is not directly related to pharmacologic alterations of the contractile state of the heart.

Proposed Course of Project: Further studies will be undertaken to determine the effect of in vivo administration of digitalis and norepinephrine on myofibrillar ATPase activity.

230

Serial No. - NHI-83

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Honors and Awards: None

Publications: None

331

Serial No. - NHI-84 (c)
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Natural History of Severe, Acquired Valvular Aortic Stenosis

Previous Serial Number: NHI-34 (c)

Principal Investigator: John Ross, Jr., M.D.

Other Investigators: Stuart Frank, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years
Total: .4
Professional: .2
Other: .2

Project Description: The natural history of patients with isolated valvular aortic stenosis, in whom the severity of obstruction has been measured, remains unknown since their history usually has been interrupted by operation. The hemodynamic, clinical findings, and clinical histories of 17 such patients, aged 28 to 59 years, who were not subjected to operative intervention, and who were followed for 2 to 12 years were analyzed. Severe aortic stenosis, without significant regurgitation, was present in all patients, the transvalvular pressure gradients being $\geq$ 50 mg Hg, or the valve indices $\leq$0.70 cm.^{2}/M^2 BSA. The duration of survival was not correlated with the age at onset of symptoms, and in the patients who died, the average life expectancy after the onset of symptoms was 4.4 years (range 0.5 to 11 years). Among the entire group followed 1 to 6 years after cardiac catheterization, 65% are dead, and of 12 patients whose status was known during a 5-year interval after study, 83% are dead. These findings provide unique information concerning the prognosis of patients with hemodynamically defined aortic stenosis and indicate that, regardless of age, the onset of symptoms portends a short life expectancy in the great majority of patients.

Proposed Course of Project: Project completed. Manuscript in preparation.

Honors and Awards: None

Publications:
ARTICLE PUBLISHED IN PERIODICAL: Ross, J., Jr., and Braunwald, E.: The influence of cardiac surgery on the natural history of aortic stenosis. In press. Circulation, Supplement (July 1968).

Serial No.- NHI-85 (c)

1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Malposition of the Mitral Leaflet During Systole: A Component of the Obstruction in Idiopathic Hypertrophic Subaortic Stenosis

Previous Serial Number: NHI-71(c)

Principal Investigator: Allan L. Simon, M.D.

Other Investigators: John Ross, Jr., M.D.
James H. Gault, M.D.

Cooperating Units: Diagnostic Radiology Department, The Clinical Center

Man Years:

Total:	.4
Professional:	.2
Others:	.2

Project Description: Considerable controversy exists concerning the angiographic anatomy of the left ventricle (LV) during systole in patients with idiopathic hypertrophic subaortic stenosis (IHSS) and intraventricular pressure gradients. LV angiocardiograms were reviewed in detail in 58 patients with IHSS. 43 studies were technically satisfactory and 36 (83%) exhibited, in addition to the usual angiographic features, a characteristic combination of abnormalities: in 83%, in the frontal projection, a linear radiolucent area extended across the LV outflow tract during systole 2 to 2.5 cm. below the aortic annulus, at a level corresponding to the site of the intraventricular pressure change; in the left oblique or lateral projections, the anterior mitral leaflet was identified in diastole, and during systole this leaflet did not retract normally but projected anteriorly. The radiolucent area in the frontal view corresponded to the leading edge of the mitral leaflet. The jet of mitral regurgitation, when present, was seen below the immobilized mitral leaflet. In addition, the axis of the papillary muscles was abnormal, being directed more anterolaterally than usual by the inferior septal muscle mass.

The following sequence is postulated: Asymmetric ventricular hypertrophy causes maldirection of the papillary muscles, producing abnormal stress on the chordae tendineae during systole and thereby restricting the posterior excursion of the mitral leaflets. Thus, the anterior leaflet is held in the outflow tract, where it meets the hypertrophied interventricular septum in midsystole. It is proposed that this mechanism plays an important role in

233

producing mechanical obstruction to ejection in many patients with idiopathic hypertrophic subaortic stenosis.

Proposed Course of Project: Project completed.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Simon, A. L., Ross, J., Jr., and Gault, J. H.: Angiographic anatomy of the left ventricle and mitral valve in idiopathic hypertrophic subaortic stenosis. Circulation 36: 852-867, 1967.

234

Serial No. - NHI-86
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Chemical Energetics of Cardiac Muscle in Hyperthyroidism

Previous Serial No.: None

Principal Investigator: C. Lynn Skelton, M.D.

Other Investigators: Peter E. Pool, M.D.
Shirley C. Seagren, B.A.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: Certain features of clinical and experimental hyperthyroidism have been considered manifestations of changes in chemical energy generation. Alterations in efficiency of energy utilization may also be important in hyperthyroidism, but this efficiency has not been measured directly. Accordingly, conversion of chemical energy to mechanical work was studied in myocardium from hyperthyroid and normal cats. Hyperthyroidism was induced by intraperitoneal injection of l-thyroxine (1 mg/Kg, 10 to 14 days). In each animal serum protein bound iodine was greater than 20 μg% (normal 5.2 $\pm$ 0.4 μg%) and serum cholesterol was 57 $\pm$ 3 mg% (normal 71 $\pm$ 5 mg%). The utilization of chemical energy ($\Delta\sim$P), consisting of creatine phosphate and ATP, was determined in isolated right ventricular papillary muscles treated with iodoacetic acid and nitrogen. Under these conditions net production of $\sim$P was inhibited. The basal rate of energy utilization in muscles resting without tension was higher in 39 muscles from hyperthyroid animals than in 76 normal muscles (0.99 vs. 0.77 μmoles $\Delta\sim$P/g/min). The efficiency of energy utilization was determined in 34 muscles from hyperthyroid animals and 28 normal muscles. After inhibition of energy production, the muscles were stretched to the peak of their previously determined length-active tension curves and stimulated to contract isometrically 25 times. Although the contractile element work performed was similar in each group (hyperthyroid: 370 $\pm$ 25 g-cm/g; normal: 371 $\pm$ 20 g-cm/g) energy utilization was significantly greater ($p < .0001$) in hyperthyroid muscles than in normals (5.56 $\pm$ 0.34 vs. 2.43 $\pm$ 0.31 μmoles $\Delta\sim$P/g/25 contractions). Energy utilization per unit work was, therefore, greater in muscles from hyperthyroid animals than normals (0.016 $\pm$ 0.001 vs. 0.007 $\pm$ 0.001 μmoles $\Delta\sim$P/g-cm contractile element work). This implies a direct effect of thyroid

hormone on mechanochemical coupling, leading to decreased efficiency in the conversion of chemical energy to mechanical work in the myocardium in hyperthyroidism.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Serial No. - NHI-87
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of Hyperthyroidism on Myocardial Oxygen Consumption

Previous Serial Number: None

Principal Investigator: C. Lynn Skelton, M.D.

Other Investigators: Henry N. Coleman, M.D.
Kern Wildenthal, M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years (computed for the 12 month period)

Total:	.8
Professional:	.4
Other:	.4

Project Description: Recent studies from this laboratory suggest that myocardial oxygen consumption ($M\dot{V}O_2$) is largely determined by developed tension and velocity of contraction. Although the intrinsic velocity of contraction and maximum isometric tension development of cardiac muscle are increased in hyperthyroidism, studies on the intact heart in man and experimental animals with hyperthyroidism have failed consistently to demonstrate an augmented $M\dot{V}O_2$. However, the complex architecture of the intact heart makes it difficult to control the factors affecting $M\dot{V}O_2$, thereby complicating attempts to quantitate differences in $M\dot{V}O_2$ between different experimental groups.

Accordingly, the present investigation was designed to study the effects of hyperthyroidism on the $M\dot{V}O_2$ of the isolated cat papillary muscle using a polarographic oxygen electrode. The mechanical behavior of the muscle was recorded simultaneously.

Cats were made hyperthyroid by the intraperitoneal injection of L-thyroxine (1 mg/kg, 10 to 14 days). Papillary muscles from hyperthyroid and normal cats were studied under isotonic and isometric conditions. As previously found in this laboratory, the contractile state of muscles from hyperthyroid cats was augmented both in terms of tension development and velocity of contraction. Comparisons of the $M\dot{V}O_2$ of isometric contractions at the apex of the length-active tension curve was inconclusive because of great variation in the level of tension development between the two groups. However, comparison of the $M\dot{V}O_2$ values for afterloaded isotonic contractions at equivalent levels of tension development revealed a higher $M\dot{V}O_2$ in the hyper-

237

thyroid group. There was no significant difference in basal $\dot{M}VO_2$ between the two groups.

These results are consistent with previous work demonstrating an increased $\dot{M}VO_2$ in association with an increase in the velocity of myocardial contraction. However, the results could also be interpreted as consistent with an inefficiency of energy utilization in hyperthyroidism.

Proposed Course of Project: Further experiments are planned to better define the mechanism of increased $\dot{M}VO_2$ in hyperthyroidism.

Honors and Awards: None

Publications: None

Serial No. - NHI-88
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Myocardial Contractile State in Hypertrophy and Failure, Studied in the Intact Heart and Isolated Muscle

Previous Serial Number: None

Principal Investigator: James F. Spann, Jr., M.D.

Other Investigators: James W. Covell, M.D.
Dwain L. Eckberg, M.D.
Edmund H. Sonnenblick, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: The intrinsic mechanical properties of isolated papillary muscles from hypertrophied and failing hearts have recently been defined. However, the level of mechanical function per unit of muscle within the intact but chronically hypertrophied and failing ventricle has not been defined.

Right ventricular (RV) hypertrophy and right heart failure were induced in cats by graded constriction of the main pulmonary artery. After hypertrophy and heart failure had been established for 4 weeks right heart and aortic pressures were determined by catheterization and cardiac outputs and heart rate were measured. Following this, the RV pressure and rate of rise of pressure (dp/dt) were determined during an isovolumic beat produced by sudden occlusion of the pulmonary artery during the preceding diastole; the right atrium was opened, a RV papillary muscle was removed for study and the passive pressure volume relations of the RV were determined. This allowed calculation of the maximum contractile element velocity of shortening and the tension developed per unit of cardiac muscle. These studies were also done in 8 normal cats.

In intact failing hearts, average RV weight increased 132%, peak isovolumic pressure 89%, and RV end-diastolic pressure from 3 to 13 cm H_2O. RV end-diastolic volume increased 95% and maximum isometric tension (P_o) 32%. However, the maximum extrapolated contractile element velocity (V_{max}) was markedly reduced, from the normal value of 2.31 to 1.39 muscle lengths/sec. In the

239

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

papillary muscles from the same ventricles, P_o at the apex of the length-tension curve and V_{max} were greatly reduced.

Thus, in heart failure, both force and velocity were depressed in the isolated muscle, while increased fiber length maintained force developed per unit of muscle in the intact ventricle. This increased preload and the greater muscle mass provided augmented total ventricular force and pressure development. However, V_{max}, which is unaltered by muscle length or mass, provided an accurate reflection of the depressed ventricular contractile state.

These findings provide a quantitative analysis of depressed intrinsic contractile state in the intact, chronically hypertrophied and failing myocardium, indicating that the rate of interaction of contractile sites may be reduced in both hypertrophy and overt heart failure. They further demonstrate the manner in which augmentation of end-diastolic volume and total muscle mass compensate for this intrinsic defect.

Proposed Course of Project: Project completed.

Honors and Awards: None

Publications: Manuscript in preparation.

Serial No. - NHI-89
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Lipid Metabolism of the Hypertrophied and Failing Heart.

Previous Serial Number: None

Principal Investigator: James F. Spann, Jr., M.D.

Other Investigators: Benjamin Wittels, M.D.

Cooperating Units: Department of Pathology,
Duke University Medical Center
Durham, North Carolina

Man Years:

Total:	.6
Professional:	.3
Other:	.3

Project Description: A number of investigations have shown that free fatty acids serve as a major source of energy for the myocardium. It has also been shown that mitochondrial function and high energy phosphate stores are intact in cardiac muscle isolated from the failing heart and thus do not appear to account for the profound depression of contractile function observed in such muscle. It was therefore of interest to examine the ability of failing heart muscle to oxidize fatty acids in order to determine if this substrate could be fully utilized as an energy source by cardiac muscle of the failing heart. The left ventricles of 7 guinea pigs with heart failure due to severe aortic constriction and of 7 normal guinea pigs were studied.

The rate of oxidation of C^{14} labeled palmitate in the homogenates of the hearts of animals with congestive failure was found to be 30 to 50% depressed below the normal values. Addition of ATP did not improve the depressed oxidation. When mitochondrial membrane integrity was impaired by detergent action the oxidation of fatty acids returned toward normal in the preparation from failing hearts. Carnitine, a myocardial constituent which serves to control the oxidation rate of long chain fatty acids in the heart was reduced to 60% of the normal value in the failing heart. In contrast to long chain fatty acid oxidation, glucose oxidation by the failing heart was not impaired.

These findings indicate that long-chain fatty acids utilization by the failing heart is altered by a defect at the level of the active transport of fatty acids into the mitochondria for oxidation.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Proposed Course of Project: Project completed.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Wittles, B., and Spann, J. F., Jr.: Defective lipid metabolism in the failing heart. J. Clin. Invest. In press.

2x2

Serial No.- NHI-90
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Actions of Ethanol on the Contractile State of the Normal and Failing Cat Papillary Muscle

Principal Investigator: James F. Spann, Jr., M.D.

Other Investigators: Dean T. Mason, M.D.
G. David Beiser, M.D.
Herman K. Gold, M.D.

Cooperating Units: None

Man Years
Total: .8
Professional: .4
Other: .4

Project Description: Despite considerable interest in the cardiocirculatory effects of ethanol (EtOH), the direct actions on ventricular myocardium have not been well understood. Effects of the clinically meaningful blood concentration range of 100 to 500 mg.% EtOH were studied on both isotonic and isometric contractions of isolated right ventricular cat papillary muscles suspended in oxygenated Krebs solution in a myograph and electrically stimulated at a frequency of 12/min.

EtOH 100 mg.% depressed the force-velocity relationship, contractile element velocity (V_{CE}) diminishing from 1.11 to 1.01 muscle lengths/sec (- 9%) at a constant load of 0.5 g/mm^2, reflecting a negative inotropic response. V_{CE} at 0.5 g/mm^2 tension was reduced -18% with 300 mg.% and -38% with 500 mg.% EtOH. Peak isometric tension declined substantially from 7.1 to 4.8 g/mm^2 (-32%) and the maximal tension following paired electrical stimulation was reduced from 10.1 to 7.6 g/mm^2 (-25%) with 500 mg.% EtOH. The rate of tension development was diminished, 41.6 to 26.4 g/mm^2/sec (-37%), while the time to peak tension was not altered with 500 mg% EtOH, thus indicating that, as in heart failure, the intensity but not the duration of the active state declined. The effects of EtOH were quantitatively similar in the 4 cats with right ventricular failure produced by constriction of the main pulmonary artery.

It is concluded that EtOH exerts a direct negative inotropic effect on heart muscle; this action is manifest even with small concentrations of the agent and may be potentially hazardous to patients with impaired cardiac function.

Proposed course of project: To be completed
Publications: None

Honors and Awards: None

Serial No. - NHI-91
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Mechanics of Left Ventricular Contraction in Animals with Chronic Left Ventricular Hypertrophy

Previous Serial Number: None

Principal Investigator: Henry M. Spotnitz, M.D.

Other Investigators: James W. Covell, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: Cardiac function in chronic congestive heart failure has been the subject of intensive study for many years. Recent experiments in this laboratory utilizing isolated right ventricular cat papillary muscle from animals with congestive failure and cardiac hypertrophy have demonstrated a marked depression of myocardial contractility. A similar depression has been observed in the intact cat heart during hypertrophy and failure. Conversely, no depression of contractility could be demonstrated in left ventricular hypertrophy secondary to a large arteriovenous fistula. The objective of the current study is to determine the effects of chronic left ventricular hypertrophy induced by aortic constriction on the mechanics of contraction in the intact canine left ventricle. Accordingly, a coarctation of the descending thoracic aorta was produced in eleven 6-week old puppies. In the present study 18 months following the banding procedure the dogs are lightly sedated and the mechanics of left ventricular contraction are determined, utilizing standard techniques for this laboratory. These data will then be compared with those obtained in a previously studied group of fifteen normal animals.

Preliminary results suggest that the animals under study average a 20% increase in left ventricular weight with a mean gradient across the coarctation of 20 mm Hg. Left ventricular pressure of over 400 mm Hg have been observed in this group during isovolumic beats, consistent with the concept that chronic hypertrophy is not associated with myocardial depression.

Proposed Course of Project: Completion of the physiologic studies. The

fixed, hypertrophied left ventricles will be subjected to electron microscopic examination of sarcomere length and microscopic examination of fiber angle orientation.

Honors and Awards: None

Publications: None

Serial No. - NHI-91
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Mechanics of Left Ventricular Contraction in Animals with Chronic Left Ventricular Hypertrophy

Previous Serial Number: None

Principal Investigator: Henry M. Spotnitz, M.D.

Other Investigators: James W. Covell, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: Cardiac function in chronic congestive heart failure has been the subject of intensive study for many years. Recent experiments in this laboratory utilizing isolated right ventricular cat papillary muscle from animals with congestive failure and cardiac hypertrophy have demonstrated a marked depression of myocardial contractility. A similar depression has been observed in the intact cat heart during hypertrophy and failure. Conversely, no depression of contractility could be demonstrated in left ventricular hypertrophy secondary to a large arteriovenous fistula. The objective of the current study is to determine the effects of chronic left ventricular hypertrophy induced by aortic constriction on the mechanics of contraction in the intact canine left ventricle. Accordingly, a coarctation of the descending thoracic aorta was produced in eleven 6-week old puppies. In the present study 18 months following the banding procedure the dogs are lightly sedated and the mechanics of left ventricular contraction are determined, utilizing standard techniques for this laboratory. These data will then be compared with those obtained in a previously studied group of fifteen normal animals.

Preliminary results suggest that the animals under study average a 20% increase in left ventricular weight with a mean gradient across the coarctation of 20 mm Hg. Left ventricular pressure of over 400 mm Hg have been observed in this group during isovolumic beats, consistent with the concept that chronic hypertrophy is not associated with myocardial depression.

Proposed Course of Project: Completion of the physiologic studies. The

fixed, hypertrophied left ventricles will be subjected to electron microscopic examination of sarcomere length and microscopic examination of fiber angle orientation.

Honors and Awards: None

Publications: None

Serial No. - NHI-92
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effects of Steady State Changes in Afterload on Myocardial Function in the Intact, Sedated Dog

Previous Serial Number: None

Principal Investigator: Henry M. Spotnitz, M.D.

Other Investigators: James W. Covell, M.D.
John Ross, Jr., M.D.

Cooperating Units: None

Man Years

Total:	.4
Professional:	.2
Other:	.2

Project Description: In isolated cardiac muscle, the extent of shortening has previously been shown to be a function of preload, afterload, and contractile state of the myocardium. In the open chest dog, previous work in this laboratory has shown that stroke volume varies inversely with afterload if contractility and preload are maintained constant. The objective of the present study is to determine the effects of steady state changes in afterload on stroke volume in the intact sedated dog and to determine the effects of reflex changes in contractility on this relationship. Six experiments have been performed in lightly sedated closed chest dogs with flow probes implanted on the ascending aorta three weeks prior to study. With heart rate maintained at 150, a balloon in the abdominal aorta is employed to produce alterations in afterload, while preload is maintained constant through rapid transfusion or reduction in circulating blood volume. Propranolol and atropine are sequentially added to the preparation in order to control reflex changes in contractility. Preliminary results indicate that stroke volume and the extent of fiber shortening are an inverse function of afterload in the lightly sedated animal.

Proposed Course of the Project: Several more experiments are required to further elucidate the relationships under consideration.

Honors and Awards: None

Publications: None

0+6

Serial No.- NHI-93
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effects of Counterpulsation on the Mechanics of Left Ventricular Contraction and Myocardial Oxygen Consumption

Previous Serial Number: None

Principal Investigator: Henry M. Spotnitz, M.D.

Other Investigators: James W. Covell, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years (computed for the 12 month period)

Total:	.8
Professional:	.4
Other:	.4

Project Description: Previous studies concerning the effects of counterpulsation on myocardial oxygen consumption ($M\dot{V}O_2$) have suggested that the fall in $M\dot{V}O_2$ is relatively less than would be predicted from pressure-time indices. Since $M\dot{V}O_2$ is more closely related to myocardial wall stress than to pressure, the effects of counterpulsation on the mechanics of left ventricular contraction and $M\dot{V}O_2$ were examined in 9 dogs utilizing synchronized counterpulsation (15 to 20 ml) through the abdominal aorta. Heart rate was controlled and cardiac output was maintained constant by right heart bypass. Counterpulsation reduced peak LV wall stress (avg. 91.3 to 71.4 g/cm^2), integrated stress (12.8 to 9.3 g-sec/cm^2), contractile element work (107.0 to 80.1 g-M/cm^2) and fiber shortening work (94.9 to 74.7 g-M/cm^2), although the extent of fiber shortening increased from 1.75 to 1.91 cm ($p<.05$). Coronary blood flow increased by 29% during counterpulsation, and remained elevated even after counterpulsation had been discontinued. $M\dot{V}O_2$ decreased during counterpulsation (avg. 8.73 to 7.84 ml/min/100 g) ($p<.01$), a reduction commensurate with the observed changes in left ventricular mechanics. These findings support the view that when contractile state is constant, $M\dot{V}O_2$ is determined by wall stress and the work of the myocardial fibers.

Proposed Course of Project: Project completed, manuscript in preparation.

Honors and Awards: None

Publications: None

Serial No.- NHI-94
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Left Ventricular Geometry: Fiber Orientation in the Canine Left Ventricle During Diastole and Systole

Previous Serial Number: NHI-95

Principal Investigators: Daniel D. Streeter, Jr., S.M.
Henry M. Spotnitz, M.D.

Other Investigators: Dali J. Patel, M.D., Ph.D.
John Ross, Jr., M.D.
Edmund H. Sonnenblick, M.D.

Cooperating Units: Clinical Biophysics Section, Cardiology Branch, NHI

Man Years:

Total:	.8
Professional:	.4
Others:	.4

Project Description: The gross architecture and ultrastructure of the contracting left ventricle (LV) of the dog heart have been analyzed, but there is little information concerning the spatial orientation of the muscle fibers during diastole and systole. Full thickness specimens 3 mm in width, extending from base to apex and across the LV free wall, were obtained in 18 dog hearts rapidly fixed in situ by perfusion with gluteraldehyde. Six hearts were arrested in diastole, 7 in systole, and 5 in dilated diastole. Fiber orientation was determined across the wall in 15 to 25 serial sections. The angle of the circumferential fibers relative to a horizontal reference plane (perpendicular to the LV major axis) exhibited a transition from an average of $-62.0 \pm 3.2^{\circ}$ (S.E.) at the epicardium, through 0° at midwall, to $+62.1 \pm 3.5^{\circ}$ at the endocardium. When this angle was plotted against wall thickness, the resulting curvilinear relations, averaged for all sampling sites, were not different statistically in the 3 groups of hearts. Integration of the component vectors in 10 segments across the wall yielded a ratio of horizontal to vertical vectors of 2.02: 1.00. Within the free wall of the left ventricle, those fibers lying closer to the apex of the heart exhibited a more vertical orientation than those lying closer to the base. The findings indicate that the LV wall is comprised of a nonlayered continuum of fibers and that average fiber orientation does not change significantly during contraction. The data should facilitate engineering analysis of LV wall stress during the cardiac cycle.

Proposed Course of Project: Completed.

Honors and Awards: None

Publications: Manuscript in preparation

Serial No. - NHI-95
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Left Ventricular Function in Experimental Aorto-Caval Fistula with Circulatory Congestion and Fluid Retention

Previous Serial Number: None

Principal Investigator: Roger R. Taylor, M.D.

Other Investigators: James W. Covell, M.D.
John Ross, Jr., M.D.

Cooperating Units: None

Man Years

Total:	.4
Professional:	.2
Other:	.2

Project Description: The mechanical properties of left ventricular contraction were described in terms of tension, velocity, length and time in closed-chest, sedated dogs in which a large aorto-caval fistula had resulted in circulatory congestion. The results were then compared with those obtained in normal dogs. Instantaneous contractile element velocity was calculated from left ventricular pressure and its first derivative during isovolumic left ventricular contractions produced by sudden occlusion of the ascending aorta during diastole. A range of ventricular end-diastolic volumes was induced and heart rate was controlled at 150/min. Wall tension (stress) was derived from ventricular pressure and volume, the latter being obtained from the press re-volume relation of the passive ventricle. Extrapolated velocity at zero tension, V_{max}, averaged 3.0 circ/sec in the normal dogs and 2.9 circ/sec in the seven dogs with an aorto-caval fistula and fluid retension; in only one of these seven animals was V_{max} below the lower limit of normal of 2.7 circ/sec. Isovolumic tension (P_o) in dogs with aorto-caval fistulae tended to be slightly greater than normal at low ventricular filling pressures, and there was no difference in P_o between the two groups of animals at high ventricular filling pressures. Time to peak pressure averaged 151 $\pm$ 6 (SE) msec (normal 139 $\pm$ 3). Left ventricular weight averaged 6.32 $\pm$ 0.23 g/kg initial body weight (normal 5.25 $\pm$ 0.56 g/kg) ($P < 0.001$), reflecting the presence of moderate ventricular hypertrophy, and ventricular internal volume at a given filling pressure was increased proportionately. Therefore, the ventricular contractile state usually was normal in the dog with a large aorto-caval fistula, and it is proposed that mechanisms for fluid retention resulting in circulatory congestion were activated because of the large hemodynamic burden despite normal myocardial contractile properties.
Proposed Course of Project: Completed

Honors and Awards: None

249

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Publications:

ARTICLE PUBLISHED IN A PERIODICAL:

Taylor, R. R., Covell, J. W., and Ross, J., Jr.: Left ventricular function in experimental aorto-caval fistula with circulatory congestion and fluid retention. J. Clin. Invest. In press.

250

Serial No. - NHI-96

1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Influence of the Thyroid State on Left Ventricular Tension-Velocity Relations in the Intact, Sedated Dog.

Previous Serial Number: None

Principal Investigator: Roger R. Taylor, M.D.

Other Investigators: John Ross, Jr., M.D.
James W. Covell, M.D.

Cooperating Units: None

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: The mechanical properties of left ventricular contraction were described in terms of tension, velocity, length and time in closed-chest, sedated normal, hypothyroid, and hyperthyroid dogs. Heart rate was controlled at 150/min, and instantaneous contractile element velocity was calculated from left ventricular pressure and its first derivative during isovolumic left ventricular contractions, produced by sudden balloon occlusion of the ascending aorta during diastole, over a range of ventricular end-diastolic volumes. Wall tension was derived from ventricular pressure and volume, the latter being obtained from the pressure-volume relation of the arrested ventricle. The hypothyroid state was associated with a displacement of the tension-velocity relation of the left ventricle downwards and to the left, with a distinct depression of extrapolated velocity at zero tension, V_{max} (hypothyroid 2.4, normal 3.0 circumferences/sec). Total isovolumic tension (P_o) was diminished less consistently. Time to peak tension was prolonged (154 msec, normal 139 msec). In the hyperthyroid state, the tension-velocity relation of the left ventricle was displaced upwards and to the right with a marked increase in V_{max} to greater than 4 circumferences/sec and a less prominent increase in P_o. The time to peak tension was reduced (80 msec). The changes in the tension-velocity relations indicate that the inotropic state of the left ventricle in the intact dog varies directly with the animal's thyroid state.

Proposed Course of Project: Project completed.

Honors and Awards: None

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

ARTICLE PUBLISHED IN A PERIODICAL:

Taylor, R. R., Covell, J. W., and Ross, J., Jr.: Influence of the thyroid state on left ventricular tension-velocity relations in the intact, sedated dog. J. Clin. Invest. In press.

Serial No.- NHI-97

1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A Comparison of the Volume-Tension Diagrams of Ejecting and Isovolumic Contractions in the Left Ventricle of the Intact, Sedated Dog.

Previous Serial Number: None

Principal Investigator: Roger R. Taylor, M.D.

Other Investigators: James W. Covell, M.D.
John Ross, Jr., M.D.

Cooperating Units: None

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: Left ventricular volume-tension relations of ejecting and isovolumic contractions were studied in nine closed-chest, sedated dogs. Isovolumic contractions were produced by sudden balloon occlusion of the ascending aorta. Instantaneous ventricular volume in ejecting beats was derived from the time integral of ascending aortic blood flow monitored by an implanted electromagnetic probe, and left ventricular end-diastolic volume was obtained from the pressure-volume relation of the passive ventricle. In 6 of 9 animals contraction in ejecting beats proceeded to a volume-tension relation at end-ejection which approximated within 3 ml the volume-tension relation at the peak of isovolumic contractions. In 3 animals the end-ejection volume-tension relation fell short of the isovolumic relation by as much as 7 ml. Since ejecting beats reached a given volume-tension relation later in time than the isovolumic contractions, the duration of active state appeared to be increased in the ejecting beats. The finding that the ventricle frequently ejects until the volume-tension relation at end-ejection closely approximates that attained by isovolumic contractions indicates that the behavior of the intact ventricle resembles that of isolated cardiac muscle in that the relation between length and tension at maximum contraction is largely independent of initial muscle length.

Proposed Course of Project. Investigation completed.

Honors and Awards: None

253

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

ARTICLE PUBLISHED IN A PERIODICAL:

Taylor, R. R., Covell, J. W., and Ross, J., Jr.: A comparison of the volume-tension diagrams of ejecting and isovolumic contractions in the left ventricle of the intact, sedated dog. Am. J. Physiol. In Press.

Serial No. - NHI-98
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effects of Decreased Aortic Compliance on Left Ventricular Performance in the Dog.

Previous Serial Number: NIH-83

Principal Investigator: Charles W. Urschel, M.D.

Other Investigators: James W. Covell, M.D.
Edmund H. Sonnenblick, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: Recent studies which have applied isolated muscle mechanics to the intact heart have demonstrated that the overall performance of the ventricle is affected by its instantaneous loading. It has been shown that ventricular wall tension and the velocity of ejection are determined by the instantaneous impedance to ejection and ventricular size during ejection. The decrease in aortic compliance which accompanies aging in man increases the instantaneous impedance to ejection throughout systole and therefore results in an increased load.

Decreased aortic compliance was simulated in dogs using a rigid bypass from aortic arch to abdominal aortic bifurcation. When flow was diverted from the normal aorta through this rigid bypass, systolic pressure rose, ejection was delayed, left ventricular end-diastolic pressure rose from an average of 5.1 to 6.0 mm.Hg while stroke volume fell slightly (11.3 to 10.5 ml). The ejection fraction fell from 0.42 to 0.35. With the elevation in LV pressure and LV size, peak wall tension rose from 121 gm/cm^2 to 143 gm/cm^2. With the higher wall tension, calculated contractile element (CE) velocity decreased, peak CE power rose 12%, and CE work increased slightly.

When the change to the non-compliant aorta was induced with preload held constant, stroke volume fell, and LV wall tension rose, as in the experiments in which preload was allowed to vary. Contractility was unchanged, judged by the isovolumic force-velocity relation. The elevation of instantaneous impedance to ejection therefore decreased external cardiac performance and added

a significant tension load to the myocardium.

Proposed Course of Project: Experiments completed.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Urschel, C. W., Covell, J. W., Sonnenblick, E. H., Ross, J., Jr., and Braunwald, E.: Effects of decreased aortic compliance on performance of the left ventricle. Am. J. Physiol. 214: 298-304, 1968.

Serial No.- NHI-99
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Myocardial Mechanics in Aortic and Mitral Valvular Regurgitation: The Concept of Instantaneous Impedance as a Determinant of the Performance of the Intact Heart

Previous Serial Number: NIH 84

Principal Investigator: Charles W. Urschel, M.D.

Other Investigators: James W. Covell, M.D.
Edmund H. Sonnenblick, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: Previous studies on the alterations in cardiovascular dynamics produced by valvular regurgitation have illustrated how these lesions affect the heart as a pump but have not described the ways in which fundamental myocardial muscle function is altered by the abnormal load. Accordingly, in 17 dogs, controlled, metered aortic (AI) or mitral (MI) valvular regurgitation was induced. With both lesions, immediate increases occurred in total stroke volume (+65%), left ventricular end-diastolic pressure (+1.6 mm Hg), and peak ejection velocity (+32%), but contractility remained unchanged, judged by identical length-active tension curves in the presence and absence of regurgitation. Regurgitation increased, by an average of 15% (AI) and 16% (MI), the rate of tension decline during ejection, because of the more rapid decline in ventricular size. Consequently, contractile element (CE) velocity was increased (control 10.4 cm/sec, AI 13.6 cm/sec, and MI 14.4 cm/sec) as were CE work and peak power. Despite unchanged contractility, the ejection fraction (SV/EDV) rose from 0.43 (control) to 0.55 (AI) ($p < 0.01$) and to 0.59 (MI) ($p < 0.01$). When regurgitation was induced while preload was held constant, directionally similar changes in all myocardial mechanical parameters were observed. The increases in CE velocity, work, and power were seen to result from the lowered instantaneous impedance to ejection, which allowed the ventricular muscle to shorten faster and further.

In conclusion, since the velocity and extent of contractile element shorten-

357

ing and therefore stroke work, ejection fraction, peak aortic flow, and stroke volume are dependent on the instantaneous load faced by the ventricle, the circulatory changes in AI and MI can be explained by a consideration of the effects of these lesions on the impedance to left ventricular ejection.

Proposed Course of Project: Project completed.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Urschel, C. W., Covell, J. W., Sonnenblick, E. H., Ross, J., Jr., and Braunwald, E.: Myocardial mechanics in aortic and mitral valvular regurgitation: The concept of instantaneous impedance as a determinant of the performance of the intact heart. J. Clin. Invest. In press.

Serial No.- NHI-100
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effects of Acute Valvular Regurgitation on the Oxygen Consumption of the Heart

Previous Serial Number: None

Principal Investigator: C. W. Urschel, M.D.

Other Investigators: J. W. Covell, M.D.
T. P. Graham, M.D.
E. H. Sonnenblick, M.D.
J. Ross, Jr., M.D.
E. Braunwald, M.D.

Cooperating Units: None

Man Years
Total: .8
Professional: .4
Others: .4

Project Description: The effects of acute, stimulated aortic and mitral regurgitation on myocardial oxygen consumption and the mechanics of left ventricular contraction were studied in the open-chest, anesthetized dog. When mitral or aortic regurgitation was induced acutely with effective stroke volume (total stroke volume less regurgitant volume) and heart rate held constant, left ventricular end-diastolic volume, total stroke volume, the ejection fraction, left ventricular wall tension, and the extent of shortening of the contractile element and the circumferential fibers all increased.

Oxygen consumption increased only moderately (14% with mitral regurgitation and 17% with aortic regurgitation) despite the increases in tension and shortening. In order to separate the relative contributions of the increments of tension and of shortening in these changes in myocardial oxygen consumption, valvular regurgitation was induced when peak ventricular wall tension was held relatively constant using an analog computer. At a constant preload, acutely induced valvular regurgitation reduces the afterload on the ventricle and thus allows a greater fraction of the contractile activity to be manifest in shortening rather than the tension developed by the myocardial fibers. Since, as re-emphasized by this investigation, the energy expended by the contractile elements in shortening the myocardial fibers is very small relative to the energy cost of stretching the series elastic component, i.e., developing tension, the total energy costs of acutely induced mitral and aortic valvular regurgitation are relatively modest, and can be explained in part by the relatively small increases

in tension which are induced.

Proposed Course of Project: Study completed

Honors and Awards: None

Publications

ARTICLE PUBLISHED IN A PERIODICAL:

Urschel, C. W., Covell, J. W., Graham, T. P., Clancy, R. L., Ross, J.,Jr., Sonnenblick, E. H., and Braunwald, E.: The effects of acute valvular regurgitation on the oxygen consumption of the canine heart. Circulation Res. In press.

Serial No.- NHI-101
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Mechanism of Sympathomimetic Action of Cyclohexylamine and Hexylamine: A New Class of Catecholamine-Releasing Agents.

Previous Serial Number: None

Principal Investigator: Andrew S. Wechsler, M.D.

Other Investigators: Gerald Glick, M.D.
Stephen Epstein, M.D.

Cooperating Units: None

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: Phenylethylamine and its hydroxylated derivatives comprise a group of important sympathomimetic amines. Many studies have shown that one group of these compounds exerts its sympathomimetic actions indirectly by causing the release of neuronally-stored norepinephrine, a second group exerts its effects by directly interacting with the effector site and a third group exerts its effects by a combination of indirect and direct actions. The aliphatic amines have also been shown to have sympathomimetic effects, but their mechanism of action has not been elucidated. The cardiac effects of the straight chain aliphatic amine, hexylamine, and the cyclic aliphatic amine, cyclohexylamine, were studied in 27 open-chest dogs anesthetized with pentabarbital. Both cyclohexylamine and hexylamine produced marked increments in right ventricular contractile force, left ventricular dp/dt, systemic pressure, and heart rate. Propranolol abolished the cardiac effects of cyclohexylamine, but a pressor response was still observed. Administration of cocaine, which interferes with the neuronal uptake of indirectly acting sympathomimetic amines, completely blocked the cardiac and peripheral pressor responses produced by cyclohexylamine and markedly attenuated the effects of hexylamine. Six dogs in which the endogenous catecholamine stores were depleted by treatment with reserpine displayed no cardiac or peripheral pressor response to cyclohexylamine. When the cardiovascular effects of cyclohexylamine and hexylamine were compared with the cardiovascular effects caused by tyramine, a known catecholamine releasing agent, tyramine was found to be 100 times more powerful than cyclohexylamine and ten times more powerful than hexylamine. Thus, the results of the present investigation provide information concerning the mechanism of action of the sympathomimetic aliphatic amines, and demonstrate that two of

the compounds in this class, cyclohexylamine and hexylamine, exert their effects indirectly by release of norepinephrine.

Proposed Course of Project: The observation that cyclohexylamine is a powerful catecholamine-releasing agent is of considerable interest since aniline, which differs from cyclohexylamine only in having an unsaturated six-membered ring, does not cause any cardiovascular changes in the same test system. Thus, a comparison of the cardiovascular actions of aniline and cyclohexylamine emphasizes the major role that hydrogenation of an unsaturated ring may play in determining the ability of a compound to function as a sympathomimetic agent. It is not possible to predict the effect of hydrogenation of some of the naturally occurring and synthetic unsaturated sympathomimetic amines upon their potency as sympathomimetic agents or upon their mechanism of sympathomimetic amines. As a first step in exploring this problem, parahydroxyclyohexylethylamine, the saturated ring form of tyramine, an indirectly acting sympathomimetic, and dihydroxycyclohexylamine, the saturated ring form of dopamine, a directly acting sympathomimetic, will be studied. By comparing the cardiovascular effects of these compounds in untreated animals, in animals given cocaine, and in animals pretreated with reserpine, an important step will be made in elucidating the structure-activity relationships of the cyclic aliphatic amines, particularly those which are hydrogenated ring forms of naturally occurring sympathomimetic amines.

Honors and Awards: None

Publications: None

Serial No.- NHI-102

1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Role of Renal Factors in the Hypertension Associated with Experimental Coarctation of the Aorta in Dogs

Previous Serial Number: None

Principal Investigator: Andrew S. Wechsler, M.D.

Other Investigators: Gerald Glick, M.D.
James Maclowry, M.D.
Glenn Lubash, M.D.

Cooperating Units: 1. Clinical Pathology Department, The Clinical Center, NIH
2. Department of Medicine, University of Maryland School of Medicine

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: Hypertension is a commonly associated finding in patients with coarctation of the aorta. The etiology of this hypertension has not been fully defined and the relative roles of mechanical, reflex and humoral factors have not been elucidated. This study was undertaken in order to investigate the status of the renin-angiotensin system in dogs with experimental coarctation of the aorta.

Eleven puppies, two to three weeks old, underwent surgical banding of the thoracic aorta distal to the left subclavian artery and studies were performed two years later. Blood pressure gradients across the coarctation were measured and angiotensin dose-response curves were obtained in each of the dogs with coarctation and also in another series of ten control dogs. Open renal biopsy was performed on each of the dogs with coarctation and the sections prepared for staining of the juxta-glomerular apparatus. Quantitative counts of the juxta-glomerular granules will be done. Prior to biopsy, each of the dogs with coarctation and the 10 control dogs had blood drawn for determination of plasma-renin levels, liver function tests, renal function tests and an hematocrit, white blood count and differential peripheral blood count.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Honors and Awards: None

Publications: None.

Serial No. - NHI-103 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Hemodynamic Effects of the Hyperviscosity Syndrome in Man.

Previous Serial Number: NIH-85(c)

Principal Investigator: Andrew S. Wechsler, M.D.

Other Investigators: Gerald Glick, M.D.
Dean Mann, M.D.
John Wunderlich, M.D.
John Fahey, M.D.

Cooperating Units: Immunology Branch, National Cancer Institute

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: The hyperviscosity syndrome is characterized by an increased blood viscosity accompanied by neurological abberrations, bleeding disorders, and extreme malaise. Mild exertion or postural changes produce lightheadedness, severe fatigue and often shortness of breath. These symptoms are generally the result of the production of large amounts of abnormal plasma proteins which results in an abnormally high blood viscosity. Patients are uniformly improved by repeated plasmaphereses and resultant lowering of blood viscosity.

The cardiovascular dynamics associated with circulating blood of high viscosity are unknown. However, major abnormalities of flow may occur, particularly through capillary beds where the normal slowing of flow results in further increases in blood viscosity, as is characteristic of non-Newtonian fluids in which viscosity is an inverse function of velocity of flow. In addition, during exercise the volume of blood passing through capillary beds, such as that in the pulmonary circulation, is considerably increased and increments in resistance and ventricular work may become very marked in the hyperviscous state. Because of these considerations, studies were undertaken in which right heart catheterization was performed before and during supine exercise in a patient with the hyperviscosity syndrome, and these studies were repeated after the blood viscosity had been reduced by plasmaphereses. Viscosity was measured at a shear rate of 1600 t^{-1} seconds and it decreased by 33% after treatment.

In the patient studied, exercise was performed at similar levels of oxygen

consumption before and after treatment. Exercise performed in the lowered viscosity state produced an increase in pulmonary arterial mean pressure 36% less than in the high viscosity state. Similarly, pulmonary vascular resistance was reduced 48%, right ventricular stroke work was reduced 25% and left ventricular stroke work was reduced 10%. Pulmonary arterial oxygen saturation increased by 44% at the same cardiac output.

Proposed Course of Project: Project completed, to be published as a case report. Manuscript in preparation.

Honors and Awards: None

Publications: None

Serial No. NHI-104
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1967

Project Title: Effects of Hyperosmolality on Mechanical Performance of Cat Papillary Muscle

Previous Serial Number: None

Principal Investigator: Kern Wildenthal, M.D.

Other Investigators: C. Lynn Skelton, M.D.
Henry Neal Coleman, M.D.

Cooperating Units: None

Man Years

Total:	.4
Professional:	.2
Other:	.2

Project Description: Although intravascular infusion of hyperosmotic solutions has become increasingly common in clinical diagnostic and therapeutic programs in recent years, the effects of increased osmolality on myocardial mechanical characteristics have not been fully defined. Accordingly, it appeared desirable to compare the mechanical characteristics of in vitro cat right ventricular papillary muscle preparations in isoosmolal bathing solutions (normal Krebs) and in hyperosmolal bathing solutions (normal Krebs + various concentrations of sucrose). Because a recent study in intact animals has suggested the possibility that hyperosmolality may significantly alter series elastic component compliance, special attention has been directed towards analyzing that possibility in vitro. Preliminary experiments have indicated that low levels of hyperosmolality (< 100 mOsm/Kg H_2O above control) cause enhancement of contractile force, and that higher levels (< 200 mOsm/Kg H_2O above control) cause depression of contractile force. Also, series elastic component compliance has been observed to decrease at the higher levels.

Proposed Course of Project: Additional experiments will be conducted to quantify the change in series elastic compliance, force development, and contractile element velocity observed with various levels of hyperosmolality. The level of hyperosmolality at which changes in series elastic compliance become mechanically significant will be determined.

Honors and Awards: None

Publications: None

Serial No. - NHI-105
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effect of Tension Maintenance on Oxygen Consumption in Cat Papillary Muscle

Previous Serial Number: None

Principal Investigator: Kern Wildenthal, M.D.

Other Investigators: Henry Neal Coleman, M.D.

Cooperating Units: None

Man Years:

Total:	.6
Professional:	.3
Other:	.3

Project Description: The tension produced during myocardial contraction is known to be a primary determinant of myocardial oxygen consumption ($M\dot{V}O_2$). It is not certain, however, to what extent the oxygen cost of tension production is the effect of the initial development of tension and to what extent it is the effect of maintenance of tension for a finite time. Accordingly, $M\dot{V}O_2$ has been measured in cat papillary muscles in vitro during normal isometric contractions and during isometric contractions which were quick-released to preload tension at various times during the course of contraction. Preliminary experiments indicate that $M\dot{V}O_2$ for quick-released isometric contractions is almost as great as that for normal isometric contractions, thus suggesting that compared to tension development, tension maintenance plays a relatively minor role in determining $M\dot{V}O_2$.

Proposed Course of Project: Approximately six additional experiments will be performed to quantify accurately the relative roles of tension development and tension maintenance in determining $M\dot{V}O_2$.

Honors and Awards: None

Publications: None

Serial No.- NHI-106 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Skin Veins as the Sole Mediator of Venomotor Reflexes in the Forearm of Man

Previous Serial Number: None

Principal Investigator: Robert Zelis, M.D.

Other Investigators: Dean T. Mason, M.D.

Cooperating Units: None

Man Years

Total:	.6
Professional:	.3
Other:	.3

Project Description: To determine the relative participation of skin and muscle capacitance beds of the forearm in venomotor reflexes, epinephrine iontophoresis was combined with forearm plethysmography so that the volume of muscle veins could be estimated simultaneously with the volume of cutaneous veins, at a constant venous pressure. With this technique not only are the cutaneous veins markedly constricted but they also are prevented from filling since skin blood flow is abolished. In 11 studies on 7 normal subjects, the venous volume in the elevated control forearm at a congesting pressure of 30 mm Hg (VV [30]) was 4.16 $\pm$ .30 (S.E.M.) cc/100 g, while in the iontophoresed arm it was 2.54 $\pm$ .31 cc/100 g. Thus, the forearm cutaneous VV [30] was 1.62 cc/100 g. With deep breathing, ice to the forehead, and leg exercise, the cutaneous VV [30] decreased 23.7% $p < .01$), 37.9% ($p < .01$), and 32.6% ($p < .02$), respectively, whereas the muscle VV [30] was not altered significantly. Similar results were observed using the isolated arm technique and a deep muscle vein. These results indicate that, in the forearm, only cutaneous veins participate in venomotor reflexes. Further, since the forearm is principally composed of skeletal muscle and the hand skin, an explanation is provided for the observation that veins of the forearm, studied as a whole, appear less reactive to stimuli than veins of the hand.

Proposed Course of Project: Project completed. Manuscript in preparation.
Honors and Awards: None
Publications: None

Serial No. - NHI-107
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Protective Effect of Ventricular Pacing in Digitalis Induced Arrhythmias.

Previous Serial Number: None

Principla Investigator: Robert Zelis, M.D.

Other Investigators: Dean T. Mason, M.D.
James F. Spann, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.4
Other:	.4

Project Description: Although paired electrical ventricular pacing overcomes digitalis induced arrhythmias in experimental animals, this procedure may be hazardous clinically. It was considered that ventricular pacing with a single stimulus might provide the same protective effect against arrhythmias produced by the glycoside. Accordingly, a transvenous pacing catheter was placed in the right ventricle of 16 dogs and ouabain infused at the rate of 1 μg/kg/min. In 9 control animals, serious ventricular arrhythmias occurred at 51.0 ± 3.6 min (S.E.M.) and death at 94.9 ± 5.9 min. In 7 additional dogs, arrhythmias occurred at 51.6 ± 3.7 min but, in contrast, were completely abolished by pacing, begun at the onset of the rhythm disorder at a rate 20% above the ventricular rate. However, the total cumulative dose of ouabain causing death was unchanged by pacing; paced animals died of ventricular fibrillation at 94.6 ± 8.2 min. Similarly, potassium infusion did not increase the lethal dose of ouabain. Thus neither pacing nor potassium altered the maximum tolerated dose of ouabain; however, arrhythmias were more effectively suppressed during ventricular pacing than during potassium administration. Thus ventricular pacing with a single electrical stimulus will effectively overcome serious digitalis induced arrhythmias.

Proposed Course of Project: Project completed. Manuscript in preparation.

Honors and Awards: None

Publications: None

Serial No. - NHI-108 (c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Abnormal Peripheral Vascular Dynamics in Primary Systemic Amyloidosis.

Previous Serial Number: None

Principal Investigator: Robert Zelis, M.D.

Other Investigators: Dean T. Mason, M.D.
Werner Barth, M.D.

Cooperating Units: National Institute of Arthritis and Metabolic Diseases

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description: Although in systemic amyloidosis there is extensive pathologic involvement of the blood vessels of many organs, little is known concerning the functional significance of this abnormality in the peripheral vascular system. Accordingly, the response of the resistance bed in the forearm to the restoration of circulation after inflow occlusion (reactive hyperemia) and to vigorous forearm exercise (active hyperemia) was measured with a strain gauge plethysmograph in 8 patients with primary amyloidosis without heart failure and 20 normal subjects. In the normal subjects peak reactive hyperemia blood flow (RHBF) after ten minutes of arterial occlusion averaged 40.8 $\pm$ 3.1 (SEM) ml/min/100 gm and peak post-exercise blood flow (EXBF) averaged 28.5 $\pm$ 3.1. The patients with systemic amyloidosis were clearly separated into two groups functionally. Three patients had striking reductions in RHBF (21.0 $\pm$ 3.7, $p < .02$) as well as EXBF (14.8 $\pm$ 3.7, $p < .02$). Furthermore, these patients all demonstrated severe infiltration of amyloid in the walls of arterioles. The remaining 5 patients with amyloidosis did not differ significantly from the normal subjects (RHBF 43.6 $\pm$ 5.0, $p > .5$) and only one of these patients had vascular infiltration of amyloid on skeletal muscle biopsy. Resting blood flow in all but one of the amyloid patients was completely normal. It is suggested that the muscle pain often experienced by patients with systemic amyloidosis is at least in part the result of claudication secondary to diseased arterioles leading to a diminished arteriole dilator capacity and compromised blood supply during exertion.
Proposed Course of Project: Project completed. Manuscript in preparation.
Honors and Awards: None

Publications: None

Serial No. - NHI-109
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Electrical Stimulation of the Carotid Sinus Nerves: A New Approach to the Treatment of Incapacitating Angina Pectoris

Principal Investigator: Eugene Braunwald, M.D.

Other Investigators: Stephen E. Epstein, M.D.
Gerald Glick, M.D.
Andrew Wechsler, M.D.
Nina S. Braunwald, M.D.
G. David Beiser, M.D.
Morris Stampfer, M.D.
Robert E. Goldstein, M.D.
Lawrence S. Cohen, M.D.

Cooperating Units: Surgery Branch, National Heart Institute

Man Years:

Total:
Professional:
Other:

Project Description: Carotid sinus massage can relieve angina pectoris and has been utilized as a diagnostic test for angina. Stimulation of the carotid sinuses abolishes angina by lowering the three most important determinants of the energy needs of the heart, i.e., ventricular pressure, heart rate and myocardial contractility. Accordingly, it was considered that electrical stimulation of the carotid sinus nerves might be of clinical value in the treatment of angina pectoris. In 10 men and 3 women, most with well documented previous myocardial infarctions and all with severe, incapacitating angina pectoris, bipolar platinum electrodes were attached to the carotid sinus nerves. The latter were connected to a subcutaneously placed radio-frequency receiving unit that the patients could activate on demand by a radio-frequency transmitter.

In seven patients studied postoperatively stimulation of the nerves lowered arterial pressure, heart rate and the pressure-rate product, a hemodynamic index related to myocardial oxygen needs, both at rest and during exertion. Anginal episodes could be abolished immediately in each of these patients by activating the stimulator, and prophylactic activation allowed them to engage in activities that otherwise were consistently associated with severe pain. Also, stimulation allowed a marked prolongation of the duration with which each patient could pedal a bicycle ergometer and increased significantly the level of work that could be tolerated before angina occurred.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968.

Of the remaining patients, 3 are still recovering from the operation and stimulation has not as yet been attempted; one patient reacts to stimulation with only minimal decreases in heart rate and arterial pressure, and two patients died shortly after operation from acute myocardial infarction.

Preliminary hemodynamic studies in 4 patients have shown that stimulation both at rest and during supine exercise causes a marked decrease in forearm vascular resistance, total peripheral resistance and arterial pressure, and smaller reductions in cardiac output and heart rate. In addition, stimulation of the carotid sinus nerves does not appear to alter venous tone as measured by the occluded limb technique, nor does it diminish the increase in venous tone that occurs with exercise.

Proposed Course of Project: We plan to evaluate more patients with incapacitating angina pectoris and the operation will be offered to those patients judged suitable. The purpose of future studies will be to try to determine if the initial beneficial results can be extended to a larger group of patients, and if these effects can be achieved in patients in whom contraindications for myocardial revascularization procedures exist.

Honors and Awards: None

ARTICLE PUBLISHED IN A PERIODICAL:

Braunwald, E., Epstein, S. E., Glick, G., Wechsler, A., and Braunwald, N. S.: Relief of angina pectoris by electrical stimulation of the carotid sinus nerves/ New Eng. J. Med. 277: 1278-1283, 1967.

Serial No. - NHI-110
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Differentiation of Obstructive and Non-Obstructive Intraventricular Pressure Differences

Previous Serial No.: None

Principal Investigator: James H. Gault, M.D.

Coinvestigators: Allan Simon, M.D.
John Ross, Jr., M.D.
Eugene Braunwald, M.D.

Cooperating Units: Diagnostic Radiology Department, Clinical Center

Man Years

Total:	.8
Professional:	.4
Other:	.4

Project Description: In patients with idiopathic hypertrophic subaortic stenosis (IHSS) the documentation of obstruction to left ventricular (LV) outflow and estimation of its severity, must be based on the measurement of an intraventricular pressure difference. Recently, it has been demonstrated in studies in experimental animals that intraventricular pressure differences can be recorded in the absence of obstruction, when the catheter tip lies within portions of the left ventricular cavity which are obliterated during systole. Further, evidence has been presented which indicates that a similar phenomenon also occurs in patients. Therefore, it is of importance to define the determinants of non-obstructive pressure differences, and to determine whether patients with such pressure differences can be distinguished from those with true obstruction to LV outflow.

The clinical and laboratory findings were examined in six patients with non-obstructive intraventricular pressure differences, resulting from catheter entrapment in the obliterated ventricular apex, and these observations were contrasted with those obtained in 19 patients with IHSS, in whom obstruction to left ventricular outflow was documented at hemodynamic study. Cardiorespiratory symptoms were reported by 5 of 6 patients with nonobstructive pressure differences, and two patients had experienced angina and syncope. While a murmur similar in character and location to that found in patients with IHSS was present in each patient with a non-obstructive pressure difference, either at rest or following exercise, the murmur generally was nonspecific, of low intensity, and was not accompanied by a thrill. The sharp, bifid arterial pulse contour and

214

paradoxical splitting of the second heart sound with respiration commonly found in patients with IHSS and obstruction were not observed in patients with non-obstructive pressure differences. The intraventricular pressure difference exhibited a similar response during the Valsalva maneuver in patients with and without obstruction, increasing from 39 to 108 mm Hg and 52 to 120 mm Hg, respectively, in the two groups. However, in patients with non-obstructive pressure differences, as in patients without LV disease, the arterial pulse pressure following a ventricular extrasystole consistently increased, while in patients with obstruction, the pulse pressure almost invariably declined. In addition, angiographic evidence of obstruction was not observed in patients with non-obstructive pressure differences. However, in 4 of 6 patients with non-obstructive pressure differences, and in two patients in whom both obstructive and non-obstructive pressure differences were observed, obliteration of the apical portions of the left ventricular cavity during systole appeared to result from asymmetric ventricular hypertrophy.

On the basis of these observations, it is proposed that asymmetric ventricular hypertrophy may be responsible for either obstruction to left ventricular outflow, or apical obliteration resulting in non-obstructive pressure differences, but that the presence or absence of obstruction largely determines the clinical and hemodynamic manifestations resulting from the asymmetric hypertrophy.

Proposed Course of Project: Project completed. Manuscript in preparation.

Honors and Awards: None

Publications: None

Serial No.- NHI-111
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Vascular Mechanics: The Relationship of Fluid Shear and Turbulence to Endothelial Cytology and the Apparent Chemical Affinity of the Intimal Interface for Evans Blue Dye, Infused Fat Emulsion, and Thrombotic Deposits

Previous Serial Number: NHI-93

Principal Investigator: Donald L. Fry, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.4
Professional:	0.9
Other	1.5

Project Description:

Objective: To study the altered apparent physical-chemical properties of an intimal surface which has been subjected to an increased hydraulic shearing stress and to relate these chemical changes to the associated changes in endothelial cell populations.

Methods Employed: Using a specially designed nontraumatic intra-aortic device to produce a wide range of hydraulic shearing stress along a segment of otherwise unmolested endothelial surface, a variety of associated cytologic and physical-chemical changes were produced. The details of this technique have been presented previously. In the present group of studies Evans Blue Dye was given the animals to form a visual tag for albumin so that areas of endothelial surface which become more permeable to albumin and presumably other serum proteins will stain blue. The serum fat level was increased by the infusion of Intralipid fat emulsion. Photographic and densimetric techniques were developed to estimate the intimal concentration of Evans Blue Dye and histologic techniques developed to estimate the population densities of normal and abnormal endothelial cells, as well as the percent surface area involved with increased fat deposition and fibrin deposition.

Major Findings: These studies confirmed and extended the observations of the previous study. An acute critical stress for endothelial cells was defined which represents that stress above which endothelial cells are transformed from essentially normal elastic bodies to deformed viscous bodies. Thus, the acute critical stress is a parameter which defines the rigidity of the endothelial surface. This value has been found to

be about 400 S.D. 80 dynes per cm^2. A second parameter defining endothelial rheologic properties is the "yield stress" of the cells which defines the stress in a given study at which the viscous cells reach their critical deformation and leave the basement membrane. The product of this yield stress times the duration of the study was shown to represent the viscous resistance of the cells to deformation, and thus may be thought of as a parameter defining the "dynamic" rigidity of the endothelial cells. For stress exposure in excess of the yield stress, the endothelial cells are totally eroded from the basement membrane, subjecting it, in turn, to the forces of the adjacent flow. Strong correlations were found between the duration of exposure of the basement membrane and the deposition of both fat and fibrin. The concentration of Evans Blue Dye in the endothelial surface also was found to have a high correlation with stress exposure.

Significance to Bio-medical Research and the Program of the Institute: These studies demonstrate clearly that acute endothelial damage even to the point of total erosion can occur from purely hydraulic forces or shearing stresses which are only slightly higher than those which can occur under extreme physiologic circumstances. Measureable parameters of the endothelial strength to resist these forces have been defined which will be tools in further experimental pathology. Similarly, measureable physico-chemical changes also have been defined which are the analogs of certain vascular disease processes.

Proposed Course of the Project: A series of studies will be designed to relate the foregoing observations progressively more closely to their disease counterparts.

Honors and Awards: None

Publications:

Fry, Donald L.: Acute endothelial changes associated with increased blood velocity gradients. Circulation Res. 22: 165-197, Feb. 1968.

Serial No.- NHI-112

1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH

Individual Project Report

July 1, 1967 through June 30, 1968

Project Title: Vascular Mechanics: Interfacial Electrochemical Potential

Previous Serial Number: NHI-91

Principal Investigator: Donald L. Fry, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.15
Professional:	0.05
Other:	0.1

Project Description:

Objective: To develop methods for studying the electrochemical potential at the blood-vessel wall interface.

Methods Employed: An assortment of different types of electrodes have been studied to determine their possible usefulness in the measurement of the electrical potential of the endothelial surface. A Bak recording electrometer, having an input impedance of 10^9 ohms, was used to sense the electrical potential from each of the electrodes. Each of the following types of electrodes were studied: Metallic silver-silver chloride, bright platinum, silver-silver chloride-potassium chloride micropipette electrodes, and silver-silver chloride-sodium chloride micropipette electrodes. Paired electrodes were used for the active and indifferent electrodes in the system. Excised fresh aortic tissue in either oxygenated heparinized blood or in oxygenated Krebs solution were studied. The indifferent electrode was placed in the solution at some point not in contact with the tissue and the active electrode was placed in a recording micrometer so that it could be advanced slowly toward the endothelial surface.

Results: Prior to contact with the endothelial surface the recorded potential varied around zero + 5 millivolts. At the instant of contact, in each case, the potential instantly dropped to a negative 10 to 20 millivolts depending on the degree of advancement into the tissue. The subsequent time course of voltage depended on the particular electrode. The voltage from the micropipettes usually remained fairly stable at a negative value. That from the larger diameter metallic electrodes demonstrated a progressive increase in the positive direction approaching baseline values.

Serial No.- NHI-112

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Significance to Bio-medical Research and the Program of the Institute: Information about the interfacial electrochemical potential of the endothelial surface and its relationship to associated hydrodynamic events is of considerable importance, both to our knowledge of vascular thrombosis and to furthering our understanding of the pathogenesis of certain arterial disease processes. In particular the electrical potential is of considerable interest since the cellular elements (e.g. platelets) and the macromolecules in the blood (e.g. lipoproteins) appear to be slightly negatively charged at the normal physiologic pH of the blood. It is the interaction of these charges with the field created by the charged endothelial surface which will determine whether there is a net electrical force driving these elements toward or away from the wall.

Proposed Course of Project: Any unique interpretation of the measured potentials is hazardous in light of the present scanty knowledge about the electrochemistry of electrodes passing from one chemical milieu into another, such is the case in the present preparation. Future efforts will be directed toward seeking collaborative assistance from consultants in surface chemistry, as well as from the Department of Physics and the Department of Chemistry at American University, both of whom have expressed interest and had experience in allied areas.

Honors and Awards: None

Publications: None

Serial No.- NHI-113
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Vascular Mechanics: Blood Velocity Profiles and Wall Shear in the Aorta and Its Major Branches

Previous Serial Number: NHI-90 and NHI-96

Principal Investigator: Dali J. Patel, M.D., Ph.D.

Other Investigators: Donald L. Fry, M.D.
S. C. Ling, Ph.D.
B. Atabek, Ph.D.

Cooperating Units: The Department of Space Science and Applied Physics
Catholic University of America

Man Years:

Total:	1.25
Professional:	0.25
Other:	1.0

Project Description:

Objective: To develop methods to measure 1) blood velocity field, 2) turbulence, and 3) the shearing stress on the vessel wall in the major arteries of dogs at every instant in a cardiac cycle.

Methods Employed: A constant-temperature, heated-film anemometer system has been adapted for the detailed study of in vivo aortic velocity fields. Two types of sensing probes were developed: 1) a velocity probe and 2) a velocity gradient or fluid shear stress probe. The sensing element consisted of a thin platinum film fused to the tip of a #20 gauge needle. As the fluid passes by this tip, heat is convected away at a rate which is a function of the velocity or the velocity gradient. These probes were evaluated for steady and pulsatile flow in rigid circular tubes using both a glycerin-water mixture and blood. In vivo studies were also made along the thoracic aortas of anesthetized dogs and pigs.

Major Findings: 1) In vitro measurements using both devices were found to agree closely with the values predicted by well-established theory. Moreover, the integrated velocity profiles that were measured correlated well with the simultaneously recorded flow values using orifice meter and electromagnetic flow meter techniques. 2) In vivo velocity measurements along the aorta indicated that the velocity profiles are blunt. The flow-pulse forms obtained by the heated-film technique were similar in magnitude and contour to those obtained simultaneously

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

from an electromagnetic flowmeter. Fully developed turbulent flow was not observed; only occasional "eddy" turbulence was found to occur in the aortic arch of the smaller dogs (i.e. less than 30 kg). Preliminary measurements of lumen surface shear stress by the shear probe have shown peak wall shear stresses that are approximately one-third that of the endothelial yield stress.

Significance to Bio-medical Research and the Program of the Institute: The method will provide a powerful tool in quantitative investigations of the vascular system. It will help assess the proper role of hydrodynamic factors in the development of arteriosclerosis and hypertension.

Proposed Course of Project: A study validating the method and its applicability to living animals has been completed (manuscript submitted to Circulation Research). The next phase of the study will consist of detailed measurements of velocity fields and wall-shear stress along the canine aorta and its major branches. Particular emphasis will be given to critical areas, e.g. branch sites in the aorta, coronary arteries and the renal arteries.

Honors and Awards: None

Publications: None

Serial No.- NHI-114
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Vascular Mechanics: Arterial Wall Properties

Previous Serial Number: NHI-94

Principal Investigator: Dali J. Patel, M.D., Ph.D.

Other Investigators: Donald L. Fry, M.D.

Cooperating Units: None

Man Years:
Total: 1.5
Professional: 0.5
Other: 1.0

Project Description:

Objective: To study the physical properties of the blood vessel walls under physiological conditions.

Methods Employed: Specially developed transducers were used to measure continuously blood vessel radius and longitudinal stress at various mean intravascular pressures in situ in dogs. The static elastic moduli describing vessel-wall properties in the 3 principal directions (circumferential, longitudinal and radial) were calculated from these data. The two underlying assumptions of incompressible wall material and elastic symmetry of the arterial segment were also tested experimentally.

Major Findings: 1) The blood vessel wall was found to be essentially incompressible. The value of the bulk modulus was 4.44×10^6 g/cm^2 and that of the volume strain, $\Delta V/V$, was 0.06%. The errors introduced in values calculated elastic moduli due to incompressibility assumption were less than 0.1%. 2) Under physiologic loading, the blood vessel showed no significant shearing strains; the values of shearing strains being an order of magnitude smaller than comparable values of longitudinal and circumferential strains. Thus the vessel demonstrated an elastic symmetry which simplifies the analysis of its elastic properties considerably. 3) At physiologic pressure the mean values for the three incremental elastic moduli in the circumferential, longitudinal and radial directions were 9,590, 6,380 and 5,800 gm/cm^2, respectively. 4) The mechanism by which vessels are tethered to the surrounding tissues were found to consist of inertial and viscoelastic components. The coefficients representing the magnitude of these components are listed in a previous publication.

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Serial No.- NHI-114

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Significance to Bio-medical Research and the Program of the Institute: The dynamic behavior of the vascular system depends critically upon the physical properties of the vessel wall and its tethering mechanism. These properties had not been adequately quantified prior to these studies.

Proposed course of project: The studies will be continued to include the dynamic anisotropic properties of the blood vessel wall.

Honors and Awards: None

Publications:

Janicki, Joseph S. and Patel, Dali J.: A force gauge for measurement of longitudinal stresses in a blood vessel *in situ*. *J. Biomechanics* 1: 19-21, Jan. 1968.

Serial No. - NHI-115
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Heart Geometry Study and Myocardial Mechanics

Previous Serial Number: NHI-95

Principal Investigator: Dali J. Patel, M.D., Ph.D.

Other Investigators: Donald L. Fry, M.D.
Henry M. Spotnitz, M.D.
John Ross, Jr., M.D.

Cooperating Units: Cardiology Branch, NHI

Man Years:

Total:	1.25
Professional:	0.25
Other:	1.0

Project Description:

Objective: This study consists of two parts: 1) To describe the distribution and orientation of muscle fibers in the wall of the left ventricle of hearts stopped and fixed in systole and diastole. 2) To correlate this anatomical data with the recorded patterns of contraction to evaluate the role of fiber orientation and distribution with mechanical events.

Methods Employed: 1) Experimental techniques have been described previously by Drs. Ross and Sonnenblick in which the dog's heart can be stopped and fixed in cardiac systole or diastole. We have studied these hearts in matched pairs for fiber orientation. The wall of the left ventricle was studied grossly for dimensions and microscopically for orientation and distribution of muscle fibers. 2) A force gauge capable of measuring myocardial tension in a living heart was developed (see publications) to be used in the second part of the study.

Major Findings: 1) The results of the anatomical study have shown that fibers are oriented in concentric shells. Thus the heart appears to be a laminated structure in which the angular orientation of the individual fibers vary as a continuous function of distance across the wall. 2) No significant difference was found in the fiber orientation pattern at the base of the left ventricle, between hearts fixed in systole and those fixed in diastole. Minor differences were observed near the apex. 3) Preliminary studies indicate the feasibility of using the aforementioned force gauge in acute physiologic preparations to measure the mean circumferential tension developed in the wall of the

left ventricle. An electrical caliper attached to this gauge can be used to evaluate the corresponding strain in the same direction.

Significance to Bio-medical Research and the Program of the Institute: These studies are essential to the establishment of a realistic contraction model for the intact heart.

Proposed Course of the Project: The anatomical studies are nearly complete and a manuscript is under preparation. The next step will be to try to estimate wall tension as a function of distance through the wall in the left ventricle of living dogs.

Honors and Awards: None

Publications:

Feigl, E. O., Simon, G. A., and Fry, D. L.: Auxotonic and isometric cardiac force transducers. J. Applied Phys. 23: 597-600, Oct. 1967.

ANNUAL REPORT
LABORATORY OF MOLECULAR DISEASES
NATIONAL HEART INSTITUTE
July 1, 1967 - July 1, 1968

The Laboratory of Molecular Diseases is concerned with the structure and function of certain proteins that are either related to fat transport and metabolism (both lipoproteins and certain enzymes) or are polypeptides that have hormonal action, specifically parathyroid hormone and thyrocalcitonin in the past several years. Diseases related to these functions are also studied and both laboratory and clinical research is carried on.

Among the accomplishments of the past year was the establishment of the sequence of thyrocalcitonin, and development of a sensitive radioimmunoassay for this hormone, the first preparation of a soluble protein from beta-lipoprotein and considerable elucidation of the configuration of this lipoprotein and of the structure both normal and an abnormal form of very low density lipoproteins. In addition, there were a number of advances in the understanding of fat transport and certain lipid storage diseases.

Thyrocalcitonin. This, the second hormone identified as a product of the thyroid gland, is mobilized in response to hypercalcemia and may have an important therapeutic as well as physiological role. Porcine thyrocalcitonin was purified to a highly homogenous state. The complete sequence of its 32 amino acid residues was then established using a variety of techniques for the structural analysis of proteins. The importance to the activity of the hormone of the intra-chain disulfide bridge and certain aromatic residues was demonstrated. Antibodies to thyrocalcitonin were prepared in guinea pigs. This was next employed, using I^{131}-thyrocalcitonin, to perfect a single or double-antibody immunoassay providing highly specific and sensitive measurement of the hormone in man and animal plasma or tissues. As little as 5 $\mu\mu$g/ml of porcine thyrocalcitonin can be detected. This assay was then used to show that, in animals, thyrocalcitonin is normally present, rises proportionately to the blood calcium and disappears rapidly when the calcium level is reduced. Preliminary studies show that thyrocalcitonin is present in thyroid tumors at much higher concentrations than in normal human thyroid. Clinical studies, in collaboratory with LCE, NHI continue.

The Structure of Lipoproteins. Beta-lipoproteins. The two major lipoproteins in plasma are alpha- and beta-lipoproteins. Both participate in transporting glycerides in very low density lipoproteins (VLDL) and chylomicrons and the role here of beta-lipoproteins (beta-LP) seems to be essential. Its lipid-free apoprotein has now been prepared in soluble form for the first time, using lipoproteins isolated by two different techniques. This apoprotein has been compared to the parent lipoprotein (and chemical

derivatives of both) by immunochemical and other techniques including circular dichroism (CD), optical rotatory dispersion (ORD), and IR spectroscopy. The following new information has been obtained: (1) The structural configuration of the protein, whether in lipoprotein, apoprotein, or after succinyl or other groups are added to the lipoprotein, consists mainly of beta-structure (pleated sheet) anti-parallel chains. In the presence of detergent or after solvent delipidation, much of the configuration is converted to random structure. The lipid appears to be of considerable importance to maintenance of configuration whenever the protein is altered. (2) The apoprotein and lipoprotein share immunochemical identity; there are differences in reactivity between them and their chemical derivatives depending on the antigenic challenge used to prepare the antibody; one of the most important findings was the lack of evidence for circulating apoprotein in either the normal or in patients with abetalipoproteinemia, an issue of considerable debate in this field; (3) ultracentrifuge data now suggest the possibility that the apoprotein may consist of two parts (chains) of unequal molecular weight. Thses "two forms" have also been separated on electrophoresis, not yet by chromatography. In the electron microscope apoprotein has been viewed as a fibrillar structure. The steps outlined above open up considerable possibilities of pursuing the normal and possibly genetic differences in primary and secondary structure of beta-lipoprotein.

VLDL. The very low density lipoproteins were shown here several years ago to contain both alpha- and beta-lipoproteins. Work in NHI ten years ago also suggested other proteins might be present, a finding leading to advancement of evidence of a third or "C protein" by a group at the University of Oklahoma. Normal VLDL has now been further differentiated by two delipidation procedures, zone electrophoresis and gel filtration to three components: alpha, beta lipoproteins, and a G-100 fraction (M. W. about 25, 000) containing neither alpha nor beta, but probably a third protein. This material is not identical to the "C protein" previously reported. Its significance may be considerable. At the same time, the VLDL isolated from patients with Type III familial hyperlipoproteinemia (a disorder previously established here) has been separated into two components, one comparable to VLDL seen in normals and other hyperlipoproteinemias, and a second consisting only of beta-lipoproteins bearing an abnormal load of glycerides. Kinetic data suggests that this "beta-VLDL" comes from the usual VLDL yet has an extremely low turnover in plasma. This beta-VLDL possibly contains a molecular defect that is the primary expression of the Type III gene.

The kinetic data mentioned above (in collaboration with NHI-LM and NIAMD investigators) are part of a study using labeling of plasma triglycerides following injection of C^{14}-free fatty acids, subsequent isolation of lipoprotein glycerides, and computer-based analysis of the time course of labeling. Both normal subjects and patients with Type III and IV hyperlipoproteinemia have been examined. The data suggest that "carbohydrate-

induction" (high carbohydrate feeding) causes an increase in conversion of fatty acids and other precursors to glyceride in the liver. Normals have a corresponding increase in removal from the plasma, Type IV patients may not, thus underlying their hyperlipemia. Studies of labeled alpha- and beta-lipoproteins in dogs have indicated the retention of label without alteration of the biological activity of the lipoproteins. Turnover studies are now underway in man.

Lipoprotein Lipase. A search for a better method of assay for lipoprotein lipase in plasma (a test for Type I hyperlipoproteinemia) has led to several interesting findings. First, this enzyme, assayed as post-heparin lipolytic activity (PHLA), is highly temperature sensitive; activity is optimally assayed at 27°. Secondly, the enzyme(s) can be extracted from plasma, thus freeing it from competition with endogenous substrate. A sensitive set of assays employing label glycerides are now being used for separation of the tri- and monoglyceridase activity appearing in plasma after heparin.

Disorders of Lipid Metabolism. The LMD is conducting study of two major types of lipid disorders, familial and acquired lipoprotein abnormalities and tissue lipid storage diseases. Extensive diagnostic facility for both has been established, including the full range of techniques for characterization of plasma lipoproteins, plasma and tissue lipids, from the least polar lipids to complex glycolipids, and including tissue culture for study of genetic traits expressed in decrease in enzyme activities.

The LMD system for classifying familial hyperlipoproteinemia by lipoprotein patterns has been widely accepted. Over 250 kindreds have now been classified and propositi extensively studied. The patterns from over 1,000 samples per week are processed; the full chemical, clinical, and genetic information is retrievable through a computer program. Patients representing a minimum of seven mutants and a larger number of genotypes are under intensive in-patient and out-patient studies. During this year developments of particular interest include the following: (1) 60 patients, representing all 5 types, were administered Atromid-S on double-blind trials. The drug has been shown to be more effective when combined with diet therapy, to be very effective in Type III, usually useful in Types IV and V, able to lower blood cholesterol modestly in most patients with Type II, and of no value in Type I. A new side-effect was observed in 8 per cent of patients: myalgia with elevations of creatine phosphokinase. Symptoms occasionally required cessation of therapy.

Also interesting was the failure of orotic acid--which obliterates hepatic, but not intestinal, production of β-lipoprotein in rats--to affect plasma cholesterol levels in normal subjects or patients with Type II (in

collaboration with NIAMD workers). A new abnormality was discovered, capable of producing a phenocopy of Type I hyperlipoproteinemia. This is a syndrome of hyperchylomicronemia and low PHLA, apparently due to heparin-resistance produced by dysglobulinemia seen in systemic lupus erythematosus and similar disorders.

In study of tissue lipidosis, the defect in sphingomyelinase in organs of patients with Niemann-Pick disease was found to be present in their skin fibroblasts grown in culture (in collaboration with NINDB workers). The fm strain of mouse was found to have "Niemann-Pick disease" but without sphingomyelinase deficiency, like Types C and D of human Niemann-Pick disease. A new method for labeling G_{m1}-gangliosides--isolated from a patient with this disease--was developed and used to study β-galactosidose in tissues.

Serial No. - NHI-118 (c)

1. Laboratory of Molecular Diseases
2. Section on Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Biochemistry and Metabolism of Plasma Lipoproteins: The Effect of Orotic Acid on Lipoprotein Metabolism.

Previous Serial Number: NHI-308

Principal Investigator: Robert I. Levy, M.D.

Other Investigators: Nanci Briggs, B.A.

Cooperating Units: Herbert Windmueller, Ph.D. (NIAMD-Nutritional-Biochemistry Section); Jarvis Seegmiller, M.D. (NIAMD-Arthritis and Rheumatism Branch)

Man Years

Total:	0.5
Professional:	0.2
Other:	0.3

Project Description:

Objectives: Little is known regarding the mechanisms for release of lipid from the liver and intestine, although a central role for the beta-lipoproteins in liver and intestinal lipid release have been postulated by our laboratory as well as others. In the past we have been able to demonstrate that the fatty liver and hypolipidemia associated with feeding 1% orotic acid (OA) to rats could be directly related to the liver's inability to synthesize or release beta-lipoprotein. After OA feeding, plasma beta-lipoprotein concentrations decreased gradually to nondetectable levels over periods of 7 to 10 days. Normal perfused livers were capable of releasing albumin, alpha and beta-lipoprotein. Livers of rats fed OA for

8 days released albumin and alpha-lipoprotein, but no beta-lipoprotein seemingly emphasizing the vehicular role of beta-lipoprotein in the transport of fat out of liver.

In the familial beta-lipoprotein deficiency state in man (Abeta-lipoproteinemia), fat cannot be reabsorbed from the intestine and chylomicrons cannot be made. Preliminary studies last year suggested that the OA rat could transport exogenous fat normally. Since the OA rat had its liver lipid release mechanism turned off, investigation of intestinal fat absorption and transport in normal and treated animals was begun, in an attempt to gain information regarding the amounts and kinds of lipid and lipoprotein synthesized in the intestine. Study of lipid transport via the thoracic duct in normal or orotic acid rat on fat-free and fat-fed diets seems to be an appropriate step toward understanding the mechanisms of intestinal and hepatic lipid release.

Methods Employed: Routine methods of studying and quantifying the lipoproteins including analytical ultracentrifugation, paper electrophoresis, and heparin manganese precipitation were used along with a battery of lipid and protein analyses and immunochemical techniques employing specific antisera to the rat alpha and beta-lipoproteins. All these methods were applied as needed to the serial sampling of plasma, intestinal lymph and hepatic perfusate. In a modified type restaining Bowman cage intestinal lymph drainage could be obtained and studied for periods up to one to two weeks following intestinal lymph duct cannulation.

A selected group of normal patients and patients with hyperuricemia undergoing evaluation of uric acid metabolism in the Arthritis Institute were observed during 7 to 14 day courses of orotic acid fed in divided doses (6 gm QD) to patients while on adenine free diets. The dosage of orotic acid and the nature of the diet was quite comparable to that producing the OA effect in rats.

Major Findings: 1) Lymph collected from intestinal lymphatic vessels from rats fed a fat-free diet had much higher concentrations of beta-lipoprotein and lower alpha-lipoprotein concentrations than found in plasma. Equally high lymph beta-lipoprotein concentrations were found in rats (fed 2% orotic acid) (OA) that had no immunochemically detectable plasma beta-lipoprotein.

2) On a fat-free diet the composition and physical-chemical properties of the lipid and lipoprotein complexes obtained from the normal or OA fed rats were essentially identical.

3) Both normal and OA rats developed thoracic duct chylomicronemia some 30 to 60 minutes after the gastric infusion of corn oil. There seemed to be no difference in the time or quality of the exogenous fat transport in the two groups of animals.

4) Much of the lymph lipid and lipoproteins could be found in the very low density fraction both on a fat free and fat fed formula. Delipidation of these very low density lipoprotein complexes in all cases revealed beta-lipoprotein in both the normal and OA rat lymph that was identical in electrophoretic mobility and immunochemical reactivity both on agarose immunoelectrophoresis and double diffusion to native plasma beta-lipoprotein.

5) Studies of control and OA fed rats with tritium-labelled fatty water revealed that on the fat free diet less than 2% of fatty acids recovered in lymph beta-lipoprotein were newly synthesized by the intestine. Evidence suggested that the fatty acids associated with the phospholipids in bile were the major source of intestinal fatty acids.

6) The rapid incorporation of intraperitoneally injected C^{14} labelled leucine into the beta and very low density lipoprotein fractions in normal and OA fed rats' intestinal lymph suggested that these proteins were actively synthesized de novo in the intestine.

All suggest that the intestine is capable of synthesizing beta-lipoproteins and that this beta-lipoprotein serves a specific role in the transport of exogenous and endogenous glyceride from the intestine.

7) The feeding of orotic acid to 5 hyperuricemic males, 3 normals and 1 patient with Type II hyperlipoproteinemia failed to lower the plasma cholesterol or beta-lipoprotein cholesterol concentrations. Over a 7-14 day period there were minor, but not significant decreases in the plasma triglyceride. Thus orotic acid fed at the dosage of 6 gm QD appeared to have effect on plasma beta-lipoprotein concentrations in man.

Significance to Bio-Medical Research and the Program of the Institute: Knowledge of the mechanisms of lipoprotein synthesis and release is vital to our understanding of normal and abnormal lipid transport. This is specifically germane to vascular diseases associated with abnormal plasma lipid concentration.

Proposed Course: Attempts will be made to further characterize the nature of the intestinal lipoproteins synthesized in the normal and OA rat intestine. This will be done in part by density gradient fractionation and analytical ultracentrifugation of the very low.density lipoproteins in intestinal lymph.

The OA rat will be used as a model system in conjunction with tail vein infusions of labelled plasma and chyle lipoproteins to study both the catabolism and possible interconversion of rat plasma lipoproteins and the pathways for exogenous and endogenous lipid clearance in the rat. Massive infusions of beta-lipoprotein into OA rats will be attempted *in vivo* to correct the OA induced fatty liver.

Publications:

Windmueller, H.G., and Levy, R.I.: Production of β-lipoprotein by intestine in the rat. *J. Bio. Chem.* In press.

Serial No. - NHI-119 (c)

1. Laboratory of Molecular Diseases
2. Sections on Lipoproteins and Molecular Diseases
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Biochemistry and Metabolism of Plasma Lipoproteins: The Structure and Genetic Control of Alpha and Beta Lipoproteins.

Previous Serial Number: NHI-307 (C)

Principal Investigators: Donald S. Fredrickson, M.D.
Robert I. Levy, M.D.
Antonio Gotto, M.D.

Other Investigators: Mariel Birnbaumer, B.A.
Elizabeth Masket, B.S., M.A.
Nanci Briggs, B.A.
Seniye Temel, B.S.

Cooperating Units: Alan Rosenthal, M.D. (NHI)-Laboratory of Kidney and Electrolyte Metabolism

Man Years

Total:	3.5	Patient Days 150
Professional:	2.0	
Other:	1.5	

Project Description:

Objectives: The structure of the plasma alpha and beta lipoproteins and their genetic control in the normal and in the inborn errors of lipoprotein metabolism have been the primary interests of this study. Understanding of the normal structure of the plasma lipoproteins and the aberrations seen in the inherited disorders promises to add greatly to our insight into the physical and chemical mechanisms involved in normal lipid transport. During this past year the investigation

has concentrated predominately on the structure of beta lipoprotein. As previously, continued interest has been maintained into the nature of the Tangier alpha lipoprotein (alpha LPT) and into the lipoproteins in Type II and III hyperlipoproteinemia.

Methods Employed: Plasma was collected from fasting normal subjects in amounts of 250-500 cc. About 3 liters of plasma was obtained from two patients with Tangier disease by repeated plasma phoresis. The alpha$_T$ lipoprotein was then isolated by a combination of heparin-manganese precipitation and ultracentrifugation at density 1.21.

Beta lipoproteins were isolated from the plasma of the normal donors by ultracentrifugation between density 1.019 and 1.063 or by an initial precipitation with heparin and manganese followed by ultracentrifugation and was then used only when immunologically pure.

Studies with the isolated beta lipoprotein and its apoprotein included, succinylation with succinic anhydrate, delipidation with ether ethanol (3:1), followed by assay for phospholipid, cholesterol and triglyceride, immunoelectrophoresis and immunochemical double diffusion experiments on Ouchterlony plates. Circular dichroism (CD) optical rotatory dispersion (ORD), infrared (IR) spectroscopy and analytical ultracentrifuge.

Major Findings: 1) Two different methods for preparing a soluble delipidated beta lipoprotein have been developed. In the past this was a major obstacle to the characterization of the protein moiety since the insolubility of the delipidated product made further study impossible. The first method involved succinylation of beta lipoprotein followed by delipidation with ether ethanol (3:1). This preparation will be subsequently referred to as succinylated-beta-apoprotein or just S-β-apoprotein.

In the second method beta lipoprotein was delipidated with ether ethanol (3:1) without succinylation and the β-apoprotein was solubilized with 60 μmole of sodium decyl-sulfate. The concentration of this detergent was reduced to 0.1 μmole by dialysis without affecting the conformation or antigenicity of the beta apoprotein.

2) The β-apoprotein and S-β-apoprotein prepared contained no deductible triglyceride or cholesterol and approximately 1% phospholipid.

3) Immunological Properites - Whereas β-apoprotein reacted well with antisera to native beta lipoprotein, S-β-apoprotein reacted weakly and inconstantly or not at all. Furthermore, β-apoprotein formed precipitin lines of immunological identity to beta lipoprotein. Antisera to β-apoprotein reacted with the normal beta lipoprotein while antisera to S-β-apoprotein usually failed to react with both beta lipoprotein and β-apoprotein. Antisera to beta apoprotein formed two precipitin lines to β-apoprotein, and did not react with the 1.21 infranate of normal plasma nor the 1.21 infranate or whole plasma of patients with abeta-lipoproteinemia.

4) Confirmation - The existence of a significant quantity of pleated sheet beta structure in beta lipoprotein was established by the presence of a single negative trough at 216-218 mu in the CD spectrum with a molar ellipticity similar to that of the B form of poly-L-lysine and by a distinct I.R. maximum at 1617-1625 cm^{-1}. The presence of a constant shoulder in the I.R. spectrum at 1680 cm^{-1} suggests that at least part of prebeta sheets B chains were arranged in an antiparallel manner.

While β-apoprotein retained evidence of beta structure, S-β-apoprotein does not and shows the spectral characteristics of a disordered structure. The addition of sodium decyl sulfate to either beta lipoprotein or β-apoprotein shifted their respective spectra to that of a disordered one.

5) Ultracentrifugal Analysis - While beta lipoprotein or succinylated beta lipoprotein sedimented as a single component in the analytical ultracentrifuge, β-apoprotein (in the presence of sodium decyl sulfate and S-β-apoprotein in the presence or absence of detergent) formed at least two distinct peaks.

6) Electron Microscopy - Negatively stained preparations of beta lipoprotein appeared as homogenous spherical particles of about 200°A in diameter when examined with the electron microscope. Preparations of β-apoprotein appeared as long fibers. No such fibrillar structures were detected with S-β-apoprotein.

7) About 40 mg of $alpha_T$ apoprotein was isolated and lyophilized at -20°. No studies of this moiety were begun this

year, however, since it was felt that more preliminary work was necessary on native alpha apoprotein.

Proposed Course: Immunological and ultracentrifugal evidence of heterogeneity of β-apoprotein may be on the basis of aggregates of different size or of different polypeptide chains. Attempts will be made to separate these components (DEAE cellulose chromatography, electrophoresis on pevicon, saccharose column chromatography) so that these forms may be further characterized with respect to amino acid analysis, N- and C-terminal analysis, molecular size and immunological reactivity. The methods which have been used to characterize the properties of normal beta lipoprotein will be applied to the investigation of beta lipoproteins of individuals with genetic disorders of lipid transport (hyperlipoproteinemias Type II, III and IV) in order to gain insight into these syndromes at a molecular level.

Further studies with the β-apoprotein will include: 1) attempts to relipidate the apoprotein using the lipid solubilized in petroleum ether or aqueous dispersions of lipid, 2) further characterization of the properties and identity of other lipoprotein apoprotein derivatives of beta lipoprotein, i.e. after acetylation, dialyzation, and amidination, 3) the relationship of the lipid and protein to the secondary, tertiary and quaternary structure of the lipoprotein will be further explored with spectroscopic and other analytical chemical techniques.

The protein moieties of the alpha lipoprotein commonly found in plasma will be further characterized by N- and C-terminal analysis, amino acid analysis and fingerprinting with a major aim at the end of the coming year to have sufficient information to characterize the $alpha_T$ lipoprotein.

Publications:

Gotto, A.M., Levy, R.I., and Fredrickson, D.S.: Human serum beta-lipoprotein: Preparation and properties of a delipidated, soluble derivative. Biochem. and Biophys. Res. Commun. 31: 151-157, April 1968.

Gotto, A.M., Levy, R.I. and Fredrickson, D.S.: Preparation and properties of an apoprotein derivative of human serum beta-lipoprotein. Lipids, In press.

Gotto, A.M., Levy, R.I., Rosenthal, A.S., Birnbaumer, M.E. and Fredrickson, D.S.: The structure and properties of human beta-lipoprotein and beta-apoprotein. Biochem. Biophys. Res. Commun. In press.

Serial No. - NHI-120 (c)

1. Laboratory of Molecular Diseases
2. Section on Lipoproteins and Molecular Diseases
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Biochemistry and Metabolism of Plasma Lipoproteins: Studies of Familial Hyperlipoproteinemia

Previous Serial Number: NHI-305 (c)

Principal Investigators:
Donald S. Fredrickson, M.D.
Robert I. Levy, M.D.
Steven H. Quarfordt, M.D.
W. Virgil Brown, M.D.
Howard R. Sloan, M.D., Ph.D.
Charles J. Glueck, M.D.
Peter O. Kwiterovich, M.D.
Antonio Gotto, M.D., Ph.D.
Terry Langer, M.D.
John LaRosa, M.D.
Antonio Jover, M.D.

Other Investigators:
Elizabeth Masket, B.S., M.A.
Nanci Briggs, B.A.
M. Elanne Smootz, B.A.
Freida Brewton, B.S.
Silverene Johnson
Nancy Priddy

Cooperating Units:
J. Stuart Soeldner, M.D. and George Cahill, M.D., Joslin Research Laboratories, Boston, Massachusetts; Frank Lindgren, Ph.D. Donner Laboratories, Berkeley, California

Man Years:

Total:	8.0	Patient Days	4,500 (estimate)
Professional:	4.0	Clinic Visits	1,500
Other:	4.0		

Project Description:

Objectives: This is the largest single project devoted to clinical investigation in the laboratory. Its aim is to classify, determine the number of responsible mutations, the nature of underlying mechanisms and develop treatment for familial excesses in blood lipid concentrations. Three years ago a new system of methodology and nomenclature for the lipid transport disorders was introduced as a standard technique for phenotyping or differentiating the hyperlipoproteinemias that would have wide practical applicability to both the clinician and the geneticists. This system has been undergoing intensive testing and refinement since that time. The objectives sought with the use of this system have been fully outlined previously. They are in brief: 1) proof that lipoprotein patterns provide more useful information than determination of lipid concentrations alone; 2) development of an analytical sequence beginning with a simple technique capable of rapidly and economically separating "normals" from "abnormals" and capable of further dividing the latter into "Types" indicating different groups of metabolic abnormalities, 3) application of the analysis to as large and representative number of patients and their families as is possible, 4) correlation of these analyses with other clinical and genetic features to further test the assumption that lipoprotein patterns are capable of distinguishing different phenotypes, and 5) expansion of techniques for determining genotypes and the basic metabolic abnormalities.

The first two objectives have been reached. Application has now proceeded to samples from over five thousand patients.

With an increased staff of physicians and automated methods for accurately determining plasma triglyceride and cholesterol concentrations coupled with an increase in Durrum electrophoretic cells to 12, the laboratory has now the capacity for screening and evaluating over 1,000 samples per month.

Samples are accepted from donors referred locally, and throughout the nation. Patients are seen in a weekly clinic that has now expanded so as to average some 30 to 50 individual patient visits weekly. Certain selected patients with familial hyperlipoproteinemia are accepted for inpatient or outpatient metabolic studies predominately drug or dietary of one to 100 weeks duration. The basic technique includes the paper electrophoretogram, employing albuminated buffer. This permits preliminary segregation of normal and abnormal patterns identified as

Types I through V. Nearly all samples are sent through cholesterol and triglyceride determinations and selected ones through quantitative lipoprotein separation (beta quantification) consisting of centrifugation at plasma density, examination of electrophoretic mobility of lipoproteins in supernatant and infranatant fractions, precipitation of all lipoproteins but high density lipoprotein, and separate quantification by cholesterol content of alpha, beta and very low density lipoproteins. On most patients while on diets high in carbohydrate glucose tolerance tests with an associated immunoreactive insulin assay is done. Post-heparin lipolytic activity (PHLA) is routinely measured. Most patients are tested as to their responsiveness to 3 or 4 basic diets: isocaloric 40-40-20 (per cent of calories from fat, carbohydrate and protein), ad-lib, high fat, and high carbohydrate diets each with a constant low cholesterol content. Every effort is made to sample all the possible blood relatives of a patient and to classify him into one or more types. Often these samples are obtained from physicians or require field collections.

Atromid-S (chlorophenoxyisobutyric ethyl ester, 2 grams per day) was given in carefully monitored inpatient and outpatient studies with strict diet and weight control. The nature of the studies varied but included double blind evaluation of drug efficacy outlined more thoroughly one year ago and evaluation of the long-term responsiveness to Atromid-S and its effect in ameliorating fat and carbohydrate intolerance in patients manifesting these transport problems. In some patients responsiveness to drug therapy was correlated with diet and weight variation. An increasing number of patients have been placed on long-term Atromid-S therapy.

Cholestyramine (Questran ^{R}) is now being evaluated in patients with Type II hyperlipoproteinemia, both on open single blind studies on inpatients and in a double blind study with two control periods of 4 weeks, alternating with the period of placebo and cholestyramine therapy.

With the collaboration of the Biometrics Branch, National Heart Institute, all of the data obtained by history, physical examination and laboratory evaluation on inpatients and outpatients has been successfully transposed onto IBM punch cards. This process has now allowed us to put all our patient data onto card and tape form with ready availability for computer analysis.

Major Findings: 1) Family studies have now been extended to more than 1,800 patients with the firm classification of over 225 different kindreds with familial hyperlipoproteinemia representing over 430 different patients. These include: 15 patients from 10 kindred with Type I, 277 patients from 109 kindred with Type II, 52 patients from 38 kindred with Type III, 96 patients from 40 kindred with Type IV, and 27 patients from 17 kindred with Type V. Over 1,000 other abnormal patient samples have been screened, but because of the presence of acquired disorders, evaluation went no further. The data continue to indicate that the first three types of hyperlipoproteinemia are fairly homogenous and distinct. No patients of Type II have appeared in Type III families or vice versa. The mounting available data gives increasing credence to the impression that Types I and III represent homozygous expression with Type I being much less common than III. Types II and IV are much more common; Type II is relatively homogeneous, Type IV, less so, both appear mainly to be heterozygous expression. Type V may be a homozygous expression of "Type IV".

Because of increases in physician awareness and the metabolic unit and the clinics of the laboratory have been oversubscribed with patients over the past 12 months. The laboratory has averaged almost 3 new kindreds with hyperlipoproteinemia per week with almost two of these representing Type II hyperlipoproteinemia over this past year. With the discovery of each new kindred, especially with the Type II and IV disorder many affected relatives are found. It is becoming increasingly apparent that the Type II and IV disorders are relatively common genetic defects in the population and that Type III hyperlipoproteinemia is not as rare a disorder as previously thought.

2) All the patient and laboratory data gathered over the last 4 years was successfully transferred and transcribed onto IBM cards for ready recall and computer processing. All new patients immediately have their family history, personal history, physical examination and laboratory findings transcribed and entered in this registry immediately after their initial evaluation. This method of data processing has relieved the laboratory of the burden of transcription and recording imposed by our previous methods for storing and retrieving data. Using the IBM cards we have been able to summarize all the important information on all the kindreds with hyperlipoproteinemia we have thus far seen into a bound registry which can be regularly revised.

3) In Types II, III and IV hyperlipoproteinemia where endogenous hypertriglyceridemia is common, glucose tolerance tests with associated immunoreactive insulin measurements, glucose and carbohydrate inducibility has been experimentally evaluated. In a group of 78 patients almost equally divided between Types II, III and IV's glucose tolerance was found in 27% of the Type II's, 40% of the Type III's and 54% of the Type IV's. The majority of the patients with the Type II disorder (81%) had normal immunoreactive insulin responses, 56% of the Type III's had normal IRI, 17% were hyperinsulinemic at 25% hyperinsulinemic. The greatest in insulin responsiveness was seen in Type IV where one-third of the patients were normal insulinemic, one-third hypoinsulinemic and one-third hyperinsulinemic. When multiple correlation coefficients were calculated between mean glucose and mean IRI during the glucose tolerance tests the delta TG or TG after carbohydrate feeding, no correlation in any of the variables were found in the Type III's. A significant correlation was found in the Type II's and IV's between increasing glucose intolerance and increasing carbohydrate inducibility. Endogenous hyperglyceridemia associated with abnormal carbohydrate inducibility was found in all the Type III's, many of the Type IV's and few, if any, of the Type II's. Many of the Type IV's were resistant to carbohydrate induction. This information demonstrated both the heterogeneity of insulin responsiveness and carbohydrate inducibility in Type IV hyperlipoproteinemia and clearly indicated that the Type IV pattern is certainly not synonymous with "carbohydrate induced hypertriglyceridemia". Plasma insulin levels did not seem to be the common denominator in any of these forms of hypertriglyceridemia.

4) Atromid-S has now been given to 60 patients with familial hyperlipoproteinemia. In two Type I patients double blinded (while both on high fat and normal fat intakes), Atromid-S had no effects on blood lipid levels. In Type II hyperlipoproteinemia in some 24 patients a mean fall of 8% in plasma cholesterol and beta lipoprotein concentrations was noted. In 17 of these patients on long-term study no further fall in plasma cholesterol was observed and there was no change in xanthomata. A cholesterol restrictive diet proved as before to be the best form of therapy in the Type II patients lowering the plasma cholesterol from ad-lib diet levels by 25-35%. In Type III hyperlipoproteinemia all 21 patients receiving Atromid-S had significant lowering of their cholesterol and triglycerides. In double blind studies the mean lowering of cholesterol was over 30% and triglyceride over 45%. In 17 patients with Type

III hyperlipoproteinemia, 14 of whom have been on Atromid-S for over 12 months, plasma cholesterol and triglyceride with combination drug and diet therapy have been maintained below 200 mg% with a complete remission of all external xanthomata. The studies have clearly shown, however, that Atromid-S or diet alone are not as effective as Atromid-S and diet together.

In some Type III's preliminary evaluation of peripheral blood flow suggests an improvement in vascular tone over a 6-9 month followup period after beginning Atromid-S therapy.

In the 7 patients with Type IV and 6 patients with Type V hyperlipoproteinemia who have thus far received Atromid-S results have been equivocal. These studies have been hampered by the normalization of the blood lipids in most Type IV's on inpatient balanced diet regimens. In four selected Type IV's Atromid-S did not seem to prevent a gross rise in triglyceride when carbohydrate inducibility was studied in a double blind manner with patients on both Atromid placebo and Atromid-S. In some patients with Type V hyperlipoproteinemia no effect has been seen with Atromid-S, in others a significant but only moderate reduction in plasma cholesterol and triglyceride has been observed. The similarity and responsiveness of the first three types of hyperlipoproteinemia is consistent with, but not necessarily confirmatory of genetic data suggesting the relative homogeneity of these types. The variable response to Atromid-S and the lack of uniform insulin responses, glucose tolerance and carbohydrate inducibility suggests that the Type IV and V patterns may represent a heterogeneous group of disorders.

5) A previously unreported acute muscular syndrome has been observed in 8 of the 60 patients we have thus far treated with Atromid-S. In two patients severe myositis with myalgia, grossly elevated serum creatine phosphokinase (CPK), aldolase, SGOT and SGPT were observed. Three other patients had elevations in CPK and borderline abnormal SGOT and SGPT while on Atromid-S. In these patients the abnormalities persisted throughout the period of Atromid-S therapy disappearing one to two weeks after cessation of therapy. In these patients no subjective findings of muscular abnormalities could be observed and in one an electromyogram at the height of the CPK elevation was entirely normal. The nature of this elevation in creatine phosphokinase is unknown, but its association with muscular symptoms suggests that this enzyme should be actively measured in all patients on long-term Atromid-S therapy along with constant re-evaluation for skeletal and cardiac muscle dysfunction.

6) In three patients homozygous for Type II, cholestyramine seems to have an additive cholesterol-lowering effect to a low cholesterol diet alone. A double blind study evaluating the efficacy of cholestyramine in the outpatient and inpatient department in heterozygotes with Type II is now underway. In a few selected patients cholestyramine was found not to interfere with absorption of Atromid-S and in a few cases it appeared that cholestyramine and Atromid-S could be added together to a low cholesterol diet to gain more effective therapy.

7) Inpatient and outpatient studies have led to the following conclusions regarding the "ideal therapeutic diet" for the hyperlipoproteinemias: 1) Type I hyperlipoproteinemia responds best to a diet low in fat, which may be supplemented with medium chain-length triglycerides. 2) In Type II hyperlipoproteinemia diets should be as low in cholesterol as possible and when possible high in polyunsaturated fats. 3) In Type III, IV and V hyperlipoproteinemia reduction to ideal body weight is very important. At ideal body weight patients with Type III hyperlipoproteinemia respond best to a diet balanced in carbohydrate and fats and low in cholesterol. In the majority of the patients with Type IV hyperlipoproteinemia a diet relatively low in carbohydrate (30-35%) and high in unsaturated fat seems most efficacious. In Type V hyperlipoproteinemia a high protein diet (30-35% of calories) coupled with a diet low in fat has produced the most striking remission in hyperlipidemia and symptoms. It is unlikely that there will ever be any one form of drug or diet therapy effective in treating all the forms of hyperlipoproteinemia. As our understanding of each type increases, it is likely that we will find more effective therapy for the individual disorders.

Significance to Bio-Medical Research: Successful reordering of the concepts and classification of blood lipid abnormalities is of considerable practical importance to physicians. Some of these disorders undoubtedly play a very important role in determination of susceptibility to atherosclerosis.

Proposed Course: The objectives outlined in the introduction will continue to be pursued with increased emphasis on more detailed biological examination of some of the "Types". Our plans will include: 1) Continued expansion of our informal cooperative ties with units desiring the use of the system. Instruction will continue to be provided to other laboratories and clinics and results exchanged with laboratories here and abroad (visitors from over 100 different laboratories came during the last year for such consultation).

2) Now that all our familial data has been placed on IBM cards for easier recall, correlations between the blood lipid abnormalities and other abnormalities, vascular disease for example, and the interrelationships between lipoprotein abnormalities, diet, weight as well as a multitude of other correlations can be tested and retested using the IBM computer. Consideration is also being given to the establishment of a familial hyperlipoproteinemia registry extending beyond NIH patients.

3) Attempts will be made as before to bring to bear as many new and different biochemical measurements as possible on the mechanisms of the disorders in the respective types. Included are: plasma insulin levels, triglyceride turnover studies, the measurement of the post-heparin lipolytic enzymes and specific structural studies on the lipoprotein proteins and lipids in the different abnormalities.

4) The striking correlation of the hyperlipoproteinemias with coronary and some cases peripheral arterial disease will continue to be evaluated. Methods will be sought to evaluate the short and long-term efficacy of drug and diet therapy on the vascular system.

5) Evaluation of the efficacy of Atromid-S and cholestyramine will be continued.

Honors and Awards: The James F. Mitchell Foundation 1968 International Award for Heart and Vascular Research.

Publications:

Levy, R.I., and Fredrickson, D.S.: The diagnoses and management of hyperlipoproteinemia. The Am. J. of Card. In Press.

Serial No. - NHI-121 (c)

1. Laboratory of Molecular Diseases
2. Section on Lipoproteins and Molecular Diseases
3. Bethesda, Maryland 20014

PHS-NIH

Individual Project Report

July 1, 1967 through June 30, 1968

Project Title: The Biochemistry of Metabolism of Plasma Lipoproteins: The Functional Roles of Alpha and Beta Lipoproteins.

Previous Serial Number: NHI-306 (c)

Principal Investigators: Robert I. Levy, M.D.
Terry Langer, M.D.
Donald S. Fredrickson, M.D.

Other Investigators: Elizabeth Masket, B.S., M.A.
Nanci Briggs, B.A.
Freida Brewton, B.S.
Silverene Johnson

Cooperating Units: Warren Strober, M.D., (NCI-Metabolism Branch)

Man Years:

Total:	2.0
Professional:	1.5
Other:	.5

Project Description:

Objectives: This is a continuing long-term project concerned with both function and metabolism of the plasma lipoprotein. Much insight has been gained into the nature, structure and stoichiometric distribution of the lipoprotein proteins in the lipoprotein spectrum. The present studies were set up to specifically observe the functional role of the lipoproteins by studying the turnover and interconversions of the lipoprotein protein moieties in the plasma.

With specific radioactive tracers on the lipoprotein pro-

teins our objectives are: 1) to determine the fractional catabolic rate, biological half-life, distribution and body pool size of the beta, alpha and very low density lipoprotein proteins in normals and in patients with hyperlipoproteinemia, 2) to determine the nature of the interconversions and exchanges of protein between the different lipoprotein classes *in vivo*, 3) to determine the effects of diet and specific pharmacological agents on the synthetic and catabolic rates of the lipoprotein proteins.

Methods Employed: The standard preparative techniques using plasmaphoresis, preparative ultracentrifugation, paper electrophoresis, immunochemical identification and beta quantification was previously described. The serum lipoproteins were isolated and concentrated from normal or dyslipoproteinemic plasma by preparative ultracentrifugation and labeled *in vitro* with I^{125} by iodine monochloride method. The I^{125} lipoproteins were isolated and purified under as sterile procedures as possible. The I^{125} lipoproteins were sterilized further by ultrafiltration pyrogen tested and then injected intravenously into appropriate animals or subjects. Serial blood samples were obtained for counting, electrophoresis, beta quantification and ultracentrifugation. In all cases the lipoproteins obtained were observed for evidence of denaturation using all available physical chemical techniques prior to reinjection. Sometimes simultaneous studies with several animals and with different doses of the labeled protein were made in order to detect the presence of denatured materials. Much of the time and effort this year was spent on finding the appropriate pH and ionic condition for selectively iodinating the lipoprotein protein moieties without iodination of the lipids. Most of the preliminary biological studies and the few studies extended to man thus far have evaluated the turnover and interconversions of the beta lipoprotein.

Major Findings: 1) *In vitro* labeling with I^{125} using a modification of the iodine monochloride technique has been found to be efficient, gentle and safe. This procedure results in the firm attachment of greater than 98% of the radioactivity to the protein moiety of the beta-lipoprotein without significant denaturation or alteration in physical and chemical properties of the molecule. Similar labeling techniques applied to the alpha lipoprotein give comparable results. The very low density lipoproteins are more difficult to iodinate. Thus far from 3 to 6% of the label is retained by the lipid portion of these highly lipidated lipoproteins.

2) Preliminary studies in the dog have established the integrity of the iodinated lipoprotein and have revealed a half-life of 28-30 hours. Screening experiments in the dog have clearly suggested that the labeled lipoprotein is comparable to the native lipoprotein. Beta lipoproteins from normal patients and patients with Type II hyperlipoproteinemia have had the same turnover time in dogs. Canine beta lipoprotein has had a half life between 38 and 40 hours.

3) Preparative ultracentrifugation of the initial injected sample and the subsequently injected samples in dog and human over a 14-day period have revealed that greater than 95% of the injected beta lipoprotein maintains its density between 1.019 and 1.063 throughout the period of the study. There was no evidence of reassociation of these lipoprotein moieties into lower or higher density lipoprotein classes. Less than 1% of the activity was present in the fraction of density greater than 1.21.

4) Initial studies with humans with Type II hyperlipoproteinemia have just been begun.

Significance to Bio-Medical Research and Program of the Institute: The physiological role of the lipoprotein proteins in lipid transport and their relationship to one another is germane to the study of vascular disease associated with abnormal concentrations of plasma lipids. These studies will help to elucidate the mechanisms of lipoprotein interconversion and degradation and will yield important sights into the mechanisms of normal lipid transport.

Proposed Course: Studies of the turnover of beta lipoprotein and of the other lipoprotein proteins will continue and the effects of dietary manipulation and drug treatment on the metabolism of the protein moiety will be investigated.

2) The methodology will be extended to study the metabolism of normal beta lipoprotein and of abnormal beta lipoprotein such as that seen in Type III hyperlipoproteinemia. The nature and interconversion of the lipoprotein proteins in all the dyslipoproteinemias will eventually be observed.

3) In experimental animals I^{125} lipoproteins will be used to study the transport of lipoproteins into lymph, blood vessels and other organs and attempts will be made to understand the sight and nature of lipoprotein degradation and the lipoprotein

proteins participation in the transport of lipid into the cells.

Publications:

None

Serial No.- NHI-122 (c)

1. Laboratory of Molecular Diseases
2. Section on Molecular Diseases
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Tissue Lipidoses: Abnormal Biochemistry in Tissue Lipid Storage Diseases

Previous Serial Number: NHI-309 (c)

Principal Investigators: Donald S. Fredrickson, M.D.
Howard R. Sloan, M.D., Ph.D.

Other Investigators: Seniye Temel, B.S.

Cooperating Units: B. William Uhlendorf, Ph.D. (BS-Laboratory of Viral Immunology)
Roscoe Brady, M.D. (NINDB-Laboratory of Neurochemistry)
John Kampine, M.D., Ph.D. (NINDB)
Carl Hansen, Ph.D. (DRS-Laboratory Aids Branch
Julian Kanfer, Ph.D. (NINDB-Laboratory of Neurochemistry

Man Years:

Total:	2.0	Patient Days	150
Professional:	1.0	Clinic Visits	40
Other:	1.0		

Project Description:

Objectives: To improve knowledge of the biochemical bases for genetically-determined tissue lipid storage diseases, as well as improve diagnosis in patients and carriers. Preliminary findings in this laboratory several years ago indicated that cells derived from bone marrow of patients with the lipidoses, Niemann-Pick disease, had an abnormal sphingomyelin content which persisted in tissue culture. This was the first example of such a phenomenon among the lipidoses. The disease offers a model of great interest for study of the lipid storage diseases,

which may be of value in the broader problem of micelles, lipoproteins, and mutations affecting lipid metabolism. The continuing availability of patients and tissues has made it possible in the last few years to parley the discovery in NINDB of a sphingomyelin-cleaving enzyme into demonstration of the probable enzyme defect in this disease. In addition, the biochemical and enzymatic derangements in several other lipid storage diseases are presently being investigated. These disorders include Gaucher's disease, sulfatide lipidosis, and several of the gangliosidoses.

Methods Employed: Patients with unknown lipid storage diseases as well as Niemann-Pick disease, are admitted for evaluation, including biopsy for chemical studies, if indicated. As defined in previous project descriptions, the techniques include cultivation of cells from bone marrow, skin, or other tissues by Dr. Uhlendorf. After suitable passages, cultures are harvested, extracted, and lipid determined by standard techniques, the most important of which is quantitative thin-layer chromatography of the tissue phospholipids. Homogenates of tissues and cell cultures are prepared for determination of the activity of an enzyme-cleaving sphingomyelin at the ceramide-phosphorylcholine linkage.

The activity of this sphingomyelin-cleaving enzyme has also been determined in tissue cultures of amniotic fluid obtained as early as the second month of gestation. Methods are now being developed to determine whether recently proposed enzymatic defects in lipid storage disorders other than Niemann-Pick disease are also perpetuated in tissue culture.

Major Findings: 1) A marked decrease in sphingomyelin cleaving enzyme was demonstrated in tissues from 10 patients with a classical infantile form of Niemann-Pick disease and in 3 patients with a juvenile-visceral form of the disorder.

2) Enzymatic analyses have demonstrated that the enzymatic defect extends to tissue cultures, and lines from a number of parent (heterozygotes).

3) A spontaneous mutant strain of mice (fm) was found last year to have a lipidosis which seems relatively similar to human Niemann-Pick disease having been studied. The sphingomyelin and cholesterol content in the thymus and liver are significantly higher in affected animals than in normals. The total phospholipid is increased, however, the content of sphingomyelin

cleaving enzyme in the liver, thymus and spleen of affected mice was not depressed when compared with control mice of the same strain. This line of mice is now well established at NIH and further studies are being actively pursued.

4) The enzymatic defect in generalized gangliosidosis has been identified as a hydrolase deficiency.

Significance to Bio-Medical Research and Program of the Institute: Tissue storage of lipids is a principal process in atheroma formation. Studies which elucidate abnormalities in lipid metabolism and which provide new means for the differentiation of biochemical defects, offer useful new approaches which may yield specific clues to the process of atherogenesis. Moreover, a perpetuation of metabolic disorders in tissue culture cells makes possible a detailed approach to the pathogenesis and control of metabolic disorders.

Proposed Course: 1) Expansion of diagnostic capabilities to include all more polar glycolipids, and all sugars and hexosamines - the methodology now being nearly complete.

2) Quantification of the sphingomyelin-cleaving enzyme in parents (heterozygotes) of patients in comparison with normals.

3) Elucidation of at least two new undescribed lipidoses and two different types of Niemann-Pick disease now under study.

4) Rigorous determination of the structure of sphingomyelin in the cultured cells; comparison of the structure with the sphingomyeling obtained from the liver and the spleen of these patients.

5) Continued study of the fm strain of mice. Particularly, attempts will be made to determine the presence of the disease at a young age and to institute therapy.

6) Determination of the defect in the primary protein structures of the abnormal sphingomyelin-cleaving enzyme in two types of Niemann-Pick disease.

7) Determination of the amniotic fluid content of the various hydrolytic enzymes apparently involved in the lipid storage disorders so that in utero diagnosis will be possible.

8) Determination of these enzymatic activities in tissue cultures derived from amniotic fluid for the same purpose.

Publications:

Fredrickson, D.S: Lipid metabolism: Physiological. In Biologic Basis of Pediatric Practice, Robert E. Cooke, Editor, New York, McGraw-Hill, 1968, pp. 968-975.

Fredrickson, D.S.: Disorders of lipid metabolism, ibid, pp. 975-989.

3,8

Serial No. - NHI-123 (c)

1. Laboratory of Molecular Diseases
2. Section on Molecular Diseases
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Biochemistry and Metabolism of Plasma Lipoprotein: Post-heparin Lipolytic Enzymes and Their Role in Normal and Abnormal Lipid Transport and Clearance

Previous Serial Number: None

Principal Investigators: Donald S. Fredrickson, M.D.
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Heiner Greten, M.D.
John C. LaRosa, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:
Professional:
Other:

Patient Days
Clinic Visits

Project Description:

Objectives: The metabolic breakdown and interconversion of very low density lipoproteins is yet incompletely understood. It has been postulated that these lipoproteins, through a series of reactions, are converted to progressively higher density lipoproteins ending with a serum alpha and beta lipoprotein. The reaction is catalyzed by a collection of lipolytic enzymes released after heparin injection and termed post-heparin lipolytic activity (PHLA). In this study our aim is to describe and understand the changes that occur in the lipoprotein spectrum after heparin injection and to further characterize the enzymes involved in these changes.

PHLA and LPL can be found in various body tissues, such as adipose tissue, arterial wall, heart tissue and possibly others. In man a deficiency state of one or more of these enzymes is characteristic of at least one of the genetically determined disorders (Type I hyperlipoproteinemia). Part of this project's aim is to find a better system to measure and characterize the post-heparin lipolytic activity and to study its physiological role. For this work a new system for assay of lipolytic activity after heparin was required.

Methods Employed: Techniques of immunoelectrophoresis paper electrophoresis, preparative ultracentrifugation, column chromatography on sephadex and sagarose were applied to characterize the lipoprotein alterations that occurred after heparin. The changes that occurred in the different lipoprotein fractions were measured by beta quantification and observed in both normal patients and patients with hyperlipoproteinemia. For standard patient studies 50 mg of heparin was used and samples were obtained serially for over a 6-hour period. For activity after small injections of heparin, a new assay method was set up using a highly purified radioactive compound, glyceroltrioleate C^{14} as substrate. The use of this compound provided a sensitive, accurate and reproducible system which allowed us to specifically measure PHLA. The system employing the labeled triolein, Triton X100, albumin and tris buffer could be incubated with active fractions and the glyendease activity measured by extracting the products of incubation and then measuring fatty acids, mono, di, and triglyceride employing thin-layer chromatography and a scintillation counter.

Major Findings: After a 50 mg dose of heparin in normals and in most patients with hyperlipoproteinemia there was a prompt fall in plasma triglyceride levels which reached a peak between one and two hours. Associated with this there was a marked fall in concentrations of very low density lipoprotein and rise in concentrations of alpha and beta lipoprotein. The rise in alpha lipoproteins reached a peak some two to three hours after the heparin dose. Associated with these changes there was a prompt rise in free fatty acids which quickly returned to the baseline by about one hour. A gross increase in the electrophoretic mobility of all the lipoproteins occurred when viewed by paper electrophoresis. With immunoelectrophoresis a new immunoprecipitin line was observed after heparin administration it increased in amount for about one hour and disappeared by three to five hours. In all patients triglyceride levels, lipoprotein patterns and lipoprotein concentrations had returned

by the end of 6 hours to baseline levels.

2) In patients with Type I hyperlipoproteinemia no change in the lipoprotein fractions fall in triglyceride or appearance of new antigen forms occurred. Plasma fatty acid levels rose slightly but the pattern by lipoprotein electrophoresis was totally unaffected regardless of whether the initial pattern showed only prebeta lipoproteins or only chylomicrons. In Tangier disease a striking fall in triglyceride and very low density lipoproteins was associated with an increase in normal density beta lipoprotein and an increase in fatty acids. In the Tangier patients, however, no new antigenic line appeared after the injection of heparin. The patterns in patients with Types II, III, IV and V hyperlipoproteinemia seem to be qualitatively like that seen in normal, though in some patients with Type V, the fall in glyceride seemed less striking.

3) The antigenetic form appearing after heparin has been isolated between density 1.063 and 1.21. It has $alpha_2$ mobility and is not precipitable by heparin and manganese solutions.

4) PHLA as well as LPL incubated in vitro could be demonstrated to produce all the electrophoretic changes observed in vivo as well as an increase in alpha and beta lipoprotein, decrease in triglyceride and increase in fatty acids. Similarly, in vitro, the appearance of a new antigenetic form could be demonstrated.

5) PHLA as well as LPL from adipose tissue could be demonstrated to be very susceptible to changes in temperature. Decreasing the incubation temperature to $27^{\circ}C$ has a stablizing effect on PHLA. At this temperature measurements could be made with zero order kinetics for more than 120 minutes; whereas, at 37° the rate of FFA released declined rapidly and ceased by 40 minutes.

6) PHLA can be shown to survive lyophilization followed by several extractions of plasma with acetone and ether. The resulting delipidated ammonium hydroxide extract can be used as an enzyme source. This might be proven useful for further evaluations of PHLA in patients whose endogenous lipids otherwise interfere with the measurement of enzyme activity.

7) PHLA and LPL from adipose tissue responded in different ways to various inhibitors such as sodium chloride, protamine sulfate and $Na_4P_2O_7$ H_2O. In all cases they both were inhibited

but PHLA was more stable than LPL.

8) The pH optimum for PHLA and LPL could be shown as previously to be 8.6. There was no evidence that any lipoprotein lipase activity could be demonstrated in plasma before the injection of heparin.

Significance to Bio-Medical Research and the Program of the Institute: The nature of the enzymes involved in lipoprotein degradation, their homogeneity and specificity is important to understanding the nature of lipoprotein catabolism. The metabolism of the very low density lipoproteins is germane to the study of vascular disease associated with abnormal concentrations of the plasma lipids.

Proposed Course: 1) Attempts will be made to isolate the immunochemically reactive material produced by the release of post-heparin activity into plasma. If it can be isolated it will be characterized further by immunoelectrophoresis and appropriate lipid and protein techniques. Further studies will be made to evaluate the *in vivo* changes produced by heparin by doing long-term heparin infusion experiments to see the effect of continued *in vivo* lypolysis on the concentrations and catabolism of the very low density alpha and beta lipoproteins.

2) More specific enzyme substrates will be synthesized so that PHLA can be more completely understood. The nature of the different enzymes released by heparin will be studied in patients with varying disorders and related to the changes *in vivo* and to the nature and mechanisms of the specific lipid transport derangements. Efforts will be made to set up the specific assay systems to characterize and then study biologically the function and role of each of the enzymes released in plasma after heparin. In some cases attempts may be made to further isolate these enzyme preparations and use them *in vitro* in further studies of the normal degradation of very low density lipoproteins.

Publications:

None

322

Serial No.- NHI-124 (c)

1. Laboratory of Molecular Diseases
2. Section on Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Biochemistry and Metabolism of Plasma Lipoproteins: The Structure and Function of the Very Low Density Lipoproteins

Previous Serial Number: None

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Cooperating Units: Daniel M. Steinberg, M.D., Ph.D. and Arthur Frank, M.D. (NHI-Laboratory of Metabolism)
Mones Berman, Ph.D. (NIAMD-Mathematical Research Branch)

Man Years:

Total:	3.0
Professional:	2.0
Other:	1.0

Project Description:

Objectives: One of the principal objectives of the laboratory's study in the lipoproteins is to reconcile the traditional views of the lipoprotein spectrum toward a more functional one. For this purpose we have used in the past four divisions to describe the lipoproteins: alpha, beta and prebeta lipoproteins and chylomicrons. This year an attempt was made to gain further

521

understanding of the normal structural and functional characteristics of the very low density lipoproteins and to gain some insight into their relationships to the other serum proteins. One of the broad objectives of the study was to investigate the metabolism of the lipoprotein triglycerides in normals and in patients with hyperlipoproteinemia in an attempt to elucidate the nature of the hypertriglyceridemia associated with increases in very low density lipoprotein that occur in many of the lipid transport disorders, and to ask the question whether this triglyceride increase could be explained as being derived from increased synthesis or decreased removal of triglycerides. After studying the normal mechanisms of triglyceride production and removal the aim was to study and define the abnormal relationships that occurred between lipid and protein in the very low density lipoproteins in patients with Type III hyperlipoproteinemia.

The nature of the apoproteins involved in the structural integrity of the very low density lipoproteins is a question of special importance, with particular reference to the aminoterminal serine and threonine residues found in very low density lipoproteins. This is related to attempts to demonstrate the presence of a "C" peptide or other polypeptide in the very low density forms different than the A and B apoprotein. In this regard studies were also made to characterize the protein moiety of the very low density lipoproteins in patients with Type III hyperlipoproteinemia.

Methods Employed: The triglyceride turnover studies were performed on normal volunteers and hyperlipidemic subjects on isocaloric and high carbohydrate diets. Ten microcuries of C^{14} palmitic acid albumin complex was injected intravenously and a series of venous blood samples obtained at intervals thereafter. The disappearance of fatty acid radioactivity from the plasma and the appearance and subsequent removal of the latter in triglycerides of the lipoprotein fractions was determined as a function of time. The data obtained was analyzed using a digital computer (IBM 360-50) with the Saam program of Dr. Mones Berman. For studies in Type III hyperlipoproteinemia the various plasma lipoproteins were isolated in the preparative ultracentrifuge overnight at serum density and analyzed with respect to their electrophoretic mobility on paper as well as starch, their lipid composition and protein content as determined by immunochemical analysis. Lipoproteins in different ultracentrifugal fractions could be determined in various steady state conditions, after induction with carbohydrate, after fasting or

intravenous lypolysis provoked by the administration of heparin. Triglycerides in these various lipoprotein fractions could be studied as above and their rate of appearance and decay in specifically different chemical fractions of very low density lipoproteins could be measured. For structural characterization of the very low density lipoprotein protein moieties preparative ultracentrifugation, pevicon, paper, agarose as well as acrylamide gel electrophoresis was used. Immunochemical identification by immunoelectrophoresis and Ouchterlony double diffusion systems were used to establish purity and further identify the lipoprotein and lipoprotein protein fractions. Gel filtration was done on 4, 6 and 8% sagarose as well as Sephadex G-100, G-200 and DEAE cellulose. Delipidation procedures included: 1) heptane extraction of lyophilized salt-free VLDL, 2) ethanol ether extraction of aqueous lipoprotein solutions, 3) ethanol ether extractions of dry salt-free heptane delipidated lipoproteins. Concentrations of samples were achieved with high yield using a pressurized Diaflo membrane chambers, N-terminal amino acid analysis was done qualitatively using the Dansyl method and quantitatively using the Fluorodinitrobenzene technique. VLDL analyzed in this way was obtained from patients with Type IV and V hyperlipoproteinemia by plasmaphoresis following one week of a diet free of fat and high in carbohydrate.

Major Findings: 1) Free fatty acids were shown to be removed with equal rapidity from the plasma of patients with hyperlipoproteinemia and from normals.

2) On a high carbohydrate diet an increased flux of free fatty acid into triglyceride synthesis could be demonstrated. An increase in the conversion of non-free fatty precursors into triglyceride was also observed on the high carbohydrate diet.

3) In the normal on a high carbohydrate diet the fractional removal rate of plasma VLDL triglyceride was increased. In preliminary studies with a few Type IV's there was a suggestion that there was no significant increase in removal rate while on carbohydrate feedings.

4) In patients with Type III hyperlipoproteinemia the very low density lipoproteins could be demonstrated to contain two electrophoretic populations of lipoproteins by starch block electrophoresis. One with a migration identical to that of normal very low density lipoproteins (α_2) the other with a beta mobility (BVLDL). The α_2 and BVLDL fractions differed from each other in both protein and lipid composition. The BVLDL contained only the beta apoprotein, α_2 VLDL as in the normal contained

both the alpha and beta apoprotein. BVLDL contained a lipid composition similar to the S_f 0-20 low density lipoproteins with the exception of a great deal more triglyceride. The α_2 VLDL from the normal and Type III was similar.

5) Induction of hypertriglyceridemia by dietary carbohydrate led in the Type III to an increase in α_2 VLDL. Caloric deprivation or the injection of heparin and release of lipolytic enzymes led initially to an acute increase in BVLDL at the expense of α_2 VLDL.

6) The turnover of the glyceride in the α_2 VLDL was similar to the glyceride turnover of normal VLDL. The removal rate of triglyceride in BVLDL was identical to that of TG in the usual density beta lipoproteins. Kinetic data supported a precursor product relationship between the α_2 VLDL and BVLDL. These observations suggested that a block in catabolism of α_2 VLDL in Type III hyperlipoproteinemia caused accumulation of beta lipoproteins burdened with unusual contents of triglyceride. The slow turnover of these complexes would lead to gross hyperglyceridemia when flux of endogenous glyceride was high. Since the BVLDL persisted when blood lipids were completely normalized by drug or diet therapy, remission seen with this therapy was probably due to decreased triglyceride output from the liver, rather than to correction of the inherant defect.

7) Further studies with normals and Type IV and V very low density lipoproteins again revealed that all neutral lipids could be extracted by serial washes with N-heptane after lyophilization in the salt-free state. Protein yields of higher than 95% could be obtained.

8) The complexes of lyophilized protein could be separated into two peaks by pevicon electrophoresis, one with beta the other with alpha mobility. The third immunochemical form could usually be demonstrated in both peaks.

9) Using sagarose 4B chromatography three peaks could be obtained, a peak in the void volume could be demonstrated to be an agregate of alpha and beta lipoprotein. This material migrated into the beta zone by pevicon electrophoresis. A second peak contained material predominately precipited by antisera to beta lipoprotein and containing N-terminal glutamic acid. In the third peak N-terminal aspartic could be demonstrated as well as immunochemical reactivity with anti-alpha-lipoprotein sera. In all peaks obtained from the sephorose columns material was

found, however, which reacted with an antisera made to delipidated very low density lipoprotein. This precipitin line did not seem to correspond to either alpha or beta lipoprotein. N-terminal, serine and threonine could be found in all the fractions isolated by either sephorose chromatography or pevicon electrophoresis.

10) Ethanol ether (3:1 v/v) delipidation of the dry salt-free powder which has previously been heptane delipidated yielded 50% of the protein immediately after extraction with Veronal buffer. Immunochemically this material contained no beta apoprotein, but two forms immunochemically compatible with delipidated alpha lipoprotein could be demonstrated as well as material reacting persistently with antisera to very low density lipoproteins. On G-100 Sephadex the alpha reactivity could be readily separated from the material that reacted with the antisera to very low density lipoproteins. This material appeared to have a molecular weight of about 25,000 and seemed to account for most of the N-terminal serine and threonine.

11) The remaining protein found after ethanol ether delipidation not immediately solubilized could be solubilized in 60 mM Decyl sulfate and had reactivity to both alpha and beta lipoprotein. Thus, three different protein forms could clearly be demonstrated, two that seemed very similar to the native alpha and beta lipoproteins, a third protein or peptide of much smaller size and with N-terminal serine and threonine was present as well.

Significance to Bio-Medical Research and the Program of the Institute: The structure and physiological role of lipoproteins is germane to the study of vascular disease associated with abnormal concentrations of plasma lipids.

Proposed Course: 1) Additional studies will be done using C^{14} labeled fatty acids to compare the catabolism of the triglyceride method moieties in normal individuals and patients with Type IV hyperlipoproteinemia on balanced diets and diets high in carbohydrate. Specific attempts will be made to compare and study the triglyceride synthesis and removal rates on the different diets.

2) Detailed structural studies of the lipids and the abnormal lipoprotein forms in Type III hyperlipoproteinemia using combined TCL and GLC will be performed. An attempt will be made to further characterize structurally the beta protein

moieties in Type III hyperlipoproteinemia both as to its affinity to lipid, its turnover rate and its basic structural conformation as compared to normal in an attempt to further understand this disorder.

3) Specific characterization of what may be a new and seemingly unique protein found in very low density lipoproteins will be continued. Ultracentrifugal electrophoretic and immunochemical techniques will be employed as well as techniques for determining molecular weight, peptide mapping, N- and C-terminal groups and amino acid analysis. The stoichiometry of the various protein moieties in the very low density lipoproteins of normals and of patients with various types of hyperlipemia will be studied using the solubilized lipid-free protein moiety.

4) Attempts will be made to specifically isolate enough of this new very low density peptide form for immunochemical studies both as to its reactivity with alpha and beta lipoprotein antisera as well as to use it to make antibodies to determine whether this moiety is normally present free in plasma.

Publications:

None

Serial No. - NHI-125 (c)
1. Laboratory of Molecular Diseases
2. Section on Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Biochemistry and Metabolism of Plasma Lipoproteins: The Lipoproteins in Acquired Disorders of Lipid Transport

Previous Serial No. NHI-310

Principal Investigators: Robert I. Levy, M.D.
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Other Investigators: Nanci Briggs, B.A.
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Cooperating Units: Robert Scheig, M.D. and Gerald Klatskin, M.D., Yale-New Haven Medical Center, New Haven, Connecticut
Werner Barth, M.D. and Allen Kaplan, M.D. (NIAMD-Arthritis and Rheumatism Branch)
Raphael Shulman, M.D. (NIAMD-Clinical Hematology Branch

Man Years:

Total:	1.5
Professional:	1.0
Other:	0.5

Project Description:

Objectives: The nature, distribution, and interaction of the lipoprotein-protein moieties in certain acquired disorders

of lipid transport specifically parenchymal and obstructive liver disease, nephrosis, pancreatitis, myxedema, and diabetic acidosis provide instances of molecular abnormalities and stresses upon fat transport mechanisms which may be of greater variety and magnitude than have yet been seen in heritable forms of familial dyslipoproteinemia. The objective of this study is to redefine the nature of the lipoprotein complexes in these disorders and to determine the specific relationships of the alpha and beta lipoproteins and other possible proteins in the concentrations, and lipid and protein composition of the lipoproteins associated with these disorders.

Serial examination of the plasma lipoproteins and lipoprotein proteins in any one patient during an acute exacerbation or defervescence of a disorder related to lipid transport and metabolism will provide a dynamic view of the interrelationships of all the lipoprotein complexes present in plasma and increase our insight into the mechanisms of lipoprotein production and clearing as well as to more clearly pinpoint the particular defect in lipid transport or metabolism involved in the disorders.

Methods Employed: The techniques employed are similar to those used in this laboratory to study familial hyperlipoproteinemia. They include preparative ultracentrifugation, paper electrophoresis, column chromatography, analyses of free and total cholesterol, phospholipids, triglyceride and delipidation, hydrolysis and analysis of proteins and immunochemical identification and quantification of lipoproteins.

By applying the above mentioned methods and techniques, the concentration, physical-chemical properties and specific protein nature of the lipoprotein complexes may be compared to those found in normal plasma. This allows differentiation of disorders associated with abnormal increases in normal lipoprotein forms from those associated with structurally abnormal lipoproteins. In collaboration with other units, histological and chemical evaluation of lipid function was done on those patients where indicated and in some cases hemological evaluation of some of the normal blood clotting factors were carried out. In some patients when indicated specific fractionation of the plasma were studied as to their effect on the activity of rat adipose tissue lipase activity and their binding to H^3 heparin.

Major Findings: 1) Twenty subjects with obstructive liver disease were studied over the past year. Hyperlipoproteinemia

was observed in every subject. Alpha-lipoprotein concentrations were variously disturbed. In 9 subjects they were decreased probably in association with parenchymal hepatic disease. In one subject they were increased with abnormal physical-chemical properties. In four subjects they were increased, but migrated as alpha lipoproteins which could be isolated using the preparative ultracentrifuge in the low density lipoprotein range, but was identical to alpha lipoprotein by immunoelectrophoresis. In 4 subjects alpha lipoproteins were almost entirely absent as determined by polyanion precipitation.

2) In these subjects alpha lipoprotein could be immunochemically recognized within the low density range although it showed abnormal electrophoretic and immunochemical properties. Paper electrophoresis of this very low density lipoprotein fraction (D 1.006 to 1.063) showed a single beta lipoprotein band when stained for lipid and an additional slow moving band could be shown to contain mainly free cholesterol and lecithin as its lipid and to be reactive with antisera to alpha lipoprotein after delipidation. In two subjects relief of obstruction and clearance of the jaundice was associated with the disappearance of this slow moving band and the appearance of increased amounts of alpha lipoproteins with normal density and other normal physical-chemical properties.

3) A patient with systemic lupus erythematosis who presented as a Type I was extensively studied. When initially viewed she had extremely low levels of lipoprotein lipase and gross chylomicronemia. Later the chylomicronemia and fat intolerance cleared moderately, but the lipase activity remained low. A specific 7S globulin fraction could be demonstrated in her plasma that was capable of binding heparin. This heparin-binding could be demonstrated both directly using tritiated heparin and indirectly using an incubated fat adipose tissue lipase system. It was subsequentially found that several patients with macroglobulinemia, myeloma and other dysglobulinemias also had low levels of lipoprotein lipase and probably exhibited this same heparin binding effect. This effect could be overcome with the injection of large amounts of heparin *in vivo* and *in vitro*. These findings all suggested a mechanism for the hyperlipidemia sometimes observed in patients with dysglobulinemia and suggested that heparin or heparin like substance might be normally involved in the release of lipoprotein lipase, since two of the patients observed had clinically insignificant fat-clearing problems.

Significance to Bio-Medical Research and the Program of the Institute: The lipoproteins carry lipid into and out of the plasma. Knowledge of their components, sites of synthesis, metabolism, and function is essential for an understanding of lipid transport. This specifically is germane to vascular diseases associated with abnormal plasma concentrations of lipid.

Proposed Course: An attempt will be made to obtain large amounts of the abnormally slow migrating low density lipoprotein component that accumulates in the plasma of some patients with obstructive liver disease. An attempt will be made to specifically demonstrate the identity of the protein moiety of this form with the normal alpha lipoprotein.

2) Attempts will be made to set up an animal model in which the extent of biliary obstruction and parenchymal damage may be altered so as to further extend the correlation between the liver, parenchymal alterations and change in lipoproteins. This animal model in conjunction with turnover studies using radioactive labeled serum lipids and lipoproteins should give us a clear indication of the mechanism of the abnormalities observed in man. Attempts will also be made to correlate the abnormal changes that occur in the different lipoprotein fractions in obstructive liver disease with the nature and degree of the kinds of lipids that accumulate. Little is known regarding the synthesis, function, or catabolism of the plasma alpha lipoproteins and a more intensive inspection of the liver's role in lipoprotein metabolism as altered by liver disease appears to be a promising area of study.

3) More detailed studies of fat tolerance and heparin resistance will be carried out in patients with low post-heparin lypolytic activity with a variety of different dysproteinemias to see how common this phenomenon of heparin binding is and what role it might play in acquired fat intolerance.

Publications:

None

Serial No. NHI - 126
1. Laboratory of Molecular Diseases
2. Section on Polypeptide Hormones
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Thyrocalcitonin, Isolation and Chemical Characterization

Previous Serial Number: 311

Principal Investigators:
John T. Potts, Jr., M.D.
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Paul Munson, Ph.D.
Philip Hirsch, Ph.D.
NIAMD
Ralph Reisfeld, Ph.D.
Armour Pharmaceutical Company
Robert Schlueter, Ph.D.

Man Years:
Total: 5
Professional: 3
Other: 2

Project Description:

Objectives: To isolate and characterize porcine thyrocalcitonin and to determine its amino acid sequence.

Methods Employed: Thyrocalcitonin was isolated originally using urea extracted material processed by preparative gel electrophoresis. Subsequently, larger amounts of acid extracted porcine thyroid tissue were purified by Sephadex gel filtration and carboxymethylcellulose chromatography. Purification was monitored by use of analytical disc gel electrophoresis and thin layer chromatography on plates of silica gel cellulose and other support media, using special stains for detection of peptides. Effluent fractions from the carboxymethylcellulose columns were tested for biological activity using the standard rat bioassay procedure and amino acid analysis after enzymic

acid hydrolysis. For analysis of amino acid sequence repetitive degradations of the hormonal peptide were performed with the phenylisothiocyanate procedure of Edman. Phenylthiohydantoin amino acids cleaved from the peptide chain were identified by thin layer chromatography, gas chromatography, and mass spectrographic analysis. Peptide fragments of the polypeptide were prepared by cleavage with trypsin, chymotrypsin, cyanogen bromide, limited acid hydrolysis in 0.03 N HCl (or 5.7 N HCl for 20 to 30 minutes), pepsin, papain, and sodium metal in liquid ammonia. Repetitive degradations were performed with leucine aminopeptidase and carboxypeptidase. During the course of this work special methods had to be developed including methods for detection of the phenylthiohydantoin amino acids on gas chromatography, the enzymic digestion procedure using hydrolysis first by papain and then by aminopeptidase M in the presence of mercaptoethanol as a reducing agent, acid hydrolysis in mercaptoethanol to protect methionine residues, thin layer chromatographic analysis using selective stains adapted from published procedures and higher cross linking for polyacrylamide gel electrophoresis coupled with special stains to detect peptides lacking cationic dye binding sites.

Major Findings: Thyrocalcitonin was purified initially using urea extracted material and preparative polyacrylamide gel electrophoresis. The active substance was shown to be a peptide consisting of 30 to 40 amino acids. The isolated peptide has a specific biological activity of 200 MRC units/mg. Insufficient material was available for detailed tests of homogeneity or to provide sufficient material for detailed structural analysis. Accordingly, larger supplies of acid extracted material were provided by Armour Pharmaceutical Company and special techniques were evolved for purifying larger amounts of material in a single fractionation. Repetitive gel filtration on Sephadex-G-50 and Sephadex-G-25 (superfine) were employed. Relative purification was monitored by thin layer chromatography and disc gel electrophoresis. Finally, careful elution conditions on carboxymethylcellulose were employed using a combination of continuous and gradient elution. The peptide was processed during final stages of column purification to a stage of constant amino acid composition and constant specific activity across the peak of eluted biologically active material. The peptide was shown to be homogeneous on disc gel electrophoresis and thin layer chromatography. Further, the peptide was shown to contain a single amino-terminal amino acid (cystine). The presence of non-covalently bound co-factors (important for biological activity) was eliminated by subjecting the purified peptide to refractionation in systems employing solvent partition or electrophoresis in 6M urea. All biological activity was shown to be coincident with the purified peptide. The presence of amino sugars, other substituted amino acids or unusual peptide linkages was eliminated by a variety of techniques of analysis of the purified peptide. It was shown that similar amino acid composition was found after enzymic digestion or acid hydrolysis. Further, direct tests for carbohydrate and iodine were negative; a substantial portion of the total Kjeldahl nitrogen of the peptide was shown to be amino acid nitrogen. It was shown that the relative content of amino acids in material processed from urea extracted or acid extracted material was essentially identical. The peptide consists of 32 amino acids with the following structural formula: Lys_0, His_1, Arg_2, Tyr_1, Asp_0, Asn_4, Thr_2, Ser_4, Glu_1, Gln_0, Pro_2, Ala_1, Gly_3, Val_1, Met_1, $Ileu_0$, Leu_4, Thr_1, Phe_3, $\frac{1}{2}$-Cys_2.

334

Detailed tests of the state of the ½cystine residue in the molecule indicated that they were present in the form of an intra-chain disulfide bond. No evidence for free thiol groups was found either by direct titration with Ellman reagent or by reaction with iodoacetic acid. After reduction with mercaptoethanol, 2 moles of free thiol were found by both the Ellman reagent and alkylation with iodoacetic acid in neutral pH. Further, it was found that the hormone was isolated as two closely related forms identical in specific biological activity or amino acid composition after acid hydrolysis. Tests revealed that the two forms of the hormone differed only in the oxidation state of the single methionine residue.

The complete amino acid sequence of the molecule was determined by a combination of techniques. Repetitive Edman degradations were performed on intact thyrocalcitonin, two tryptic peptides of the molecule and heptapeptide resulting from cyanogen bromide cleavage. The order of all 32 amino acids was established by this technique. High yields of the individual amino acid were obtained as phenylthiohydantoin derivatives and the amino acid derivatives were identified by multiple techniques, principally gas chromatography. A second independent method of determining the amino acid sequence involved the preparation and isolation of 61 peptide fragments resulting from the various methods of cleavage of the polypeptide chain. The composition of these peptides was in complete agreement with the composition based on the Edman degradation. Further, the distribution of cleavage was such that a unique solution of the amino acid sequence could be deduced. It was in complete agreement with that based on the Edman data.

Initial studies aimed at characterizing the region of the polypeptide important for biological activity have established that the methionine residue may be oxidized or alkylated without loss of biological activity. On the other hand, breakage of the disulfide bond which constitutes a 1-7 terminal disulfide loop (a 23 membered ring) destroys biological activity. Further, alkylation of the tryptophan residue with 2-hydroxy-5-nitrobenzyl-bromide also essentially destroys biological activity.

Significance to Heart Research: This work is part of the continuing effort of the laboratory to gain a greater understanding of the structure and function of protein molecules, particularly polypeptide hormones. The work, in general, is of a highly basic and theoretical nature, with obvious bearings on tissue metabolism in general and the control of calcium and other ions.

Proposed Course of Research: It is planned to initiate a systematic study of the regions of the molecule important for biological and immunological activity by preparing a series of derivatives modified selectively through limited cleavage or by selective reaction of individual amino acid residues with chemical reagents. Collaborative efforts have begun with several groups to undertake synthesis of the polypeptide chain. Once synthesis has been accomplished a variety of analogs will be prepared and tests will be made to detect enhanced or reduced biological activity as result of selective alteration of the amino acid sequence or various functional groups on individual amino acid residues. It is planned further to isolate and determine the amino acid composition of

thyrocalcitonin from the bovine and ovine species.

Publications: Dr. Potts has been invited to present much of this work at the International Symposium on Protein and Polypeptide Hormones at Liege, Belgium in May and at the International Congress of Endocrinology in Mexico City in July.

1. Potts, J.T, Jr., Reisfeld, R.A., Hirsch. P.F., Cooper, C.W., Wasthed, A.B., and Munson, P.L.: Current Status of the Purification of Thyrocalcitonin. Proceedings of the Fifth Symposium on Calcified Tissues, 1967, (in press).

2. Potts, J.T., Jr., Reisfeld, R.A., Hirsch, P.F., Cooper, C.W., Wasthed, A.B., and Munson, P.L.: Purification of Porcine Thyrocalcitonin. Proc. Nat. Acad. Sci. 59: 328-335, 1967.

3. Potts, J.T., Jr., Reisfeld, R.A., Hirsch, P.F., Cooper, C.W., Wasthed, A.B., and Munson, P.L.: Purification and Characterization of Porcine Thyrocalcitonin. In, "Thyrocalcitonin and the C-cells". Taylor, S. (ed.), Wm. Heinemann, London, (in press).

4. Potts, J.T., Jr., Brewer, H.B., Jr., Reisfeld, R.A., H rsch. P.F., Schlueter, R., and Munson, P.L.: Isolation and Chemical Properties of Porcine Thyrocalcitonin. In, "Parathyroid Hormone and Thyrocalcitonin, (Calcitonin)". Talmage, R.V. and Belanger, L.F. (eds.) Excerpta Medica, p. 308-319, 1968.

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336

Serial No. NHI - 127
1. Laboratory of Molecular Diseases
2. Section on Polypeptide Hormones
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunoassay for Thyrocalcitonin

Previous Serial Number: None

Principal Investigators: Leonard J. Deftos, M.D.
Michael R. Lee, M.D.
John T. Potts, Jr., M.D.

Other Investigators:

Cooperating Units:

Man Years:

Total:	1.5
Professional:	1.5
Other:	0

Patient Days: None

Project Description:

Objectives: To develop a sensitive radioimmunoassay technique to permit the measurement of thyrocalcitonin in the blood of man and other mammalian species.

Methods Employed: Guinea pigs have been injected with 20 to 50 mg of highly purified (15-50 MRC U/mg) thyrocalcitonin. The hormone was suspended in complete Freund's adjuvant and injected subcutaneously in the thigh of guinea pigs used for immunization. Completely homogeneous thyrocalcitonin was labelled with I-131 by standard techniques and purified by adsorption to and elution from shallow columns of cellulose powder using dilutions of plasma obtained from surgically thyroidectomized patients. Plasma from animals was screened for capacity to bind I-131-labelled thyrocalcitonin. Binding by specific gamma globulin fraction in the immunized animals was shown by serum electrophoresis of immune plasma with added I-131-labelled thyrocalcitonin or by precipitation of I-131-labelled thyrocalcitonin and gamma globulin using either 50% ammonium sulfate or anti-guinea pig gamma globulin antiserum. Separation of antibody bound from free thyrocalcitonin was accomplished using chromatoelectrophoresis and dextran-coated charcoal. Estimation of the quantity of thyrocalcitonin in samples of plasma or tissue extracts was determined by incubation of appropriate dilutions of these samples with antiserum at a final dilution of 1:50,000 to 1:250,000 and 10,000 cpm of I-131-labelled thyrocalcitonin. Quantitation of the concentration of hormone present in unknown samples was made by comparison with the

standard curves in which increasing amounts of standard unlabelled thyrocalcitonin were used to displace I-131-labelled thyrocalcitonin from antibody.

Major Findings: It was found that a successful radioimmunoassay for thyrocalcitonin had been developed. The assay was selective, specific and extremely sensitive. As little as 5 uug/ml of porcine thyrocalcitonin could be detected. There was no interference or cross-reaction in the assay system by a variety of peptide hormones or crude protein fractions that were tested. Excellent agreement was shown between the results of the immunoassay and bioassay of thyrocalcitonin preparations even though preparations were tested that differed 1000-fold in specific biological activity. Tests with plasma or extracts of thyroid glands indicated that the immunoassay was capable of detecting human, porcine, bovine, and rabbit thyrocalcitonin.

Physiologic tests were performed in a series of normal and thyroidectomized rabbits. It was shown that thyrocalcitonin is circulating normally in the blood of unstimulated rabbits, the concentration being 0.14 mug/ml $\pm$ 0.7. Following infusion of calcium there was a prompt increase in thyrocalcitonin concentration in plasma; increases of 3 to 15-fold higher than the resting concentration were detected within 10 minutes of initiating calcium infusion. Increasing the rate of calcium infusion and therefore the extent of calcium challenge led to proportionate increases in the concentration of thyrocalcitonin found in blood. No immunoassayable thyrocalcitonin was found in the blood of thyroidectomized rabbits before, during, or after infusion with calcium. On the contrary, .5 to 2.0 ug/g of thyrocalcitonin was found in the excised thyroid glands after extraction. After injection of large quantities of porcine thyrocalcitonin in the rabbit it was possible by obtaining repetitive plasma samples to estimate the rate of disappearance of the peptide from blood. Disappearance half time was shown to be quite rapid (as has been found with a variety of other peptide hormones); T-1 $\frac{1}{2}$ was 5 to 12 minutes. Analysis was made of the control of secretion of thyrocalcitonin in the rabbit by contrasting the concentrations of calcium and thyrocalcitonin in plasma samples obtained during infusion with calcium. A highly proportional response was shown; there was a linear direct relationship between the concentration of blood calcium and thyrocalcitonin.

These studies in the rabbit represented the first opportunity to fully assess the hormonal character of thyrocalcitonin; the results strongly attest to the importance of thyrocalcitonin as a hormone in man and other mammals. It is now evident that thyrocalcitonin circulates normally in blood, rises rapidly in response to hypercalcemic challenge, and disappears rapidly once the physiologic stimulus is withdrawn. Preliminary tests have shown that thyrocalcitonin is present in extractsof medullary carcinoma of the thyroid in concentrations 100-fold higher than those found in normal human thyroid glands. This confirms the impression that this tumor consists of thyrocalcitonin secreting cells. Taken in conjunction with recent reports that increased concentrations of thyrocalcitonin can be detected by bioassay of peripheral blood in these patients the findings suggest that medullary carcinoma of the thyroid represents a primary hormonal excess syndrome.

Significance to Heart Research: This work is part of the continuing effort os the laboratory to gain a greater understanding of the biosynthesis of and secretion as well as the function of polypeptide hormones. The work is of considerable interest in elucidating the finely regulated control of hormone secretion, and, in turn, its regulation of calcium homeostasis, bone structure, and tissue metabolism in general.

Proposed Course of Research: It is hoped to further apply the radioimmunoassay technique to a systematic study of the factors that control secretion of the hormone in mammalian species, the mode of control of hormone secretion, and the relative importance of thyrocalcitonin and parathyroid hormone in the control of blood calcium and bone metabolism. Further improvements in the sensitivity of the assay should permit a systematic study of the concentration of thyrocalcitonin in cows (in which it is already possible by radioimmunoassay techniques employed in the laboratory to measure the concentration of circulating parathyroid hormone). Preliminary experiments indicate that a simultaneous assay will be possible for both hormones using I-125 labelling for parathyroid hormone and I-131 labelling for thyrocalcitonin. Such studies would provide a fuller appreciation of reciprocal changes in concentration of the two hormones with changing blood calcium concentrations and thus help to define the role of the two hormones in calcium homeostasis. Tests by the radioimmunoassay should help to define the presumed chemical similarity between mammalian thyrocalcitonin and ultimobranchial calcitonin thereby helping to clarify the phylogenetic significance of the hormone.

It seems likely that compensatory over-production of thyrocalcitonin plays an important role in various diseases in man particularly those characterized by hypercalcemia. Application of the assay technique should provide a detailed understanding of this compensatory mechanism and may explain the variable clinical course of certain disease states such as hyperparathyroidism. Further, the ability to detect the porcine peptide should allow systematic analysis of the blood levels achieved after various schedules of parenteral administration of thyrocalcitonin and thus serve as a useful guide in programs now underway to evaluate the therapeutic efficacy of the hormone in control of hypercalcemic states and diseases characterized by demineralization of bone. Finally, the role of thyrocalcitonin in diseases such as medullary carcinoma of the thyroid may be clarified.

Publications: Dr. Potts has been invited to present much of this work at the International Symposium on Protein and Polypeptide Hormones at Liege, Belgium in May and at the International Congress of Endocrinology in Mexico City in July.

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339

3. Potts, J.T., Jr., Deftos, L.J., and Lee, M.R.: Radioimmunoassay of Parathyroid Hormone and Thyrocalcitonin. Hospital Medicine. (in press).

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370

Serial No. NHI - 128
1. Laboratory of Molecular Diseases
2. Section on Polypeptide Hormones
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Measurement of Plasma Concentrations of Hormone in Man and Various Mammalian Species by Radioimmunoassay; Evaluation of the Control of Hormone Secretion in Physiological and Pathological States

Previous Serial Number: 313

Principal Investigators: John T. Potts, Jr., M.D.
Leonard J. Deftos, M.D.

Other Investigators: Richard Buckle, M.D.
Michael Lee, M.D.
Richard Reitz, M.D.
J.L.H. O'Riordan, M.D.

Cooperating Units: NIAMD
Gerald Aurbach, M.D.
The Veterinary School of the University of Pennsylvania, New Bolton Center, Kennett Square, Pennsylvania
Charles Ramberg, D.V.M.
Pat Mayer, D.V.M.
David Kronfeld, D.V.M., Ph.D.

Man Years
Total: 4
Professional: 3
Other: 1

Objectives: To apply the radioimmunoassay technique to the measurement of parathyroid hormone concentrations in peripheral bovine plasma, in the venous effluent from perfused parathyroid glands of goats and sheep, and in plasma samples from human subjects with various diseases involving abnormalities in calcium or bone metabolism. These studies are designed to investigate the factors controlling the secretion of parathyroid hormone, the response of the gland to various stimuli, and the nature of the abnormalities in parathyroid hormone production that characterize various disease states such as hyperparathyroidism, pseudo-hypoparathyroidism,"ectopic hyperparathyroidism". and chronic renal disease.

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Methods Employed: The radioimmunoassay technique for parathyroid hormone originally developed in collaboration with Berson and Yalow was extended to more sensitive conditions by use of non-equilibrium conditions and labeling of parathyroid hormone with I-125 to provide more stable radioactive tracer. Working with the group at the Veterinary School of the University of Pennsylvania, we studied serial plasma samples from normal animals, cows with parturient paresis ("Milk Fever"), and animals given infusions of calcium, EDTA or other ions to alter parathyroid hormone secretion.

Patients with hyperparathyroidism, ectopic hyperparathyroidism (patients with non-parathyroid malignancy whose tumors produce excessive quantities of parathyroid hormone) pseudo-hyperparathyroidism, and chronic renal disease were studied; samples were obtained from these patients not only under basal conditions but also during infusions of calcium and ethylene diaminetetra-acetic acid (EDTA).

As part of the effort to more accurately analyze parathyroid hormone production in man, a systematic study has been undertaken to evaluate the immunologic equivalence of human and bovine parathyroid hormone and to provide an immunoassay reference standard whereby human parathyroid values could be expressed in absolute rather than in bovine equivalent units. For this purpose, the capacity of exactly equivalent quantities of pure human and bovine parathyroid hormone to displace I-131-labelled bovine parathyroid hormone from antibody were compared. A somewhat less purified form of the human hormone, more stable, was then standardized against the highly purified human preparation and used thereafter to serve as an assay reference standard.

Major Findings:

1. Application of the radioimmunoassay techniques to measurement of parathyroid hormone in the plasma of the cow, goat, and sheep has proven quite successful and permitted a quite detailed investigation of the nature of the factors which control parathyroid hormone secretion, the speed and rapidity of changes in hormone concentration, and the survival time of the hormone in plasma. It has been shown that calcium is a specific and direct regulator of parathyroid hormone production. Plasma calcium controls parathyroid hormone concentration within narrow limits, hormone secretion varying inversely with serum calcium. A highly statistically significant correlation was present between plasma calcium (independent variable) and parathyroid hormone concentration (dependent variable). It was clear that the hormone is secreted continuously and it was possible to calculate the secretion rate of parathyroid hormone and the rate of biosynthesis necessary to maintain the observed production rate of the hormone. The parathyroid glands unlike many other endocrine organs exhibit only very limited storage of hormone. The rate of bio-synthesis necessary for maximal secretion is equivalent to a complete turnover of hormone every few minutes - bio-synthesis is limiting for secretion. The influence of other ions on parathyroid hormone production was investigated; plasma magnesium seems to influence parathyroid hormone production in a fashion similar to that exhibited by calcium, but plasma phosphate appears to have no direct affect on parathyroid hormone secretion.

342

2. In normal cows five to six-fold increases of parathyroid hormone concentration occur following maximal stimulation with EDTA (lowering of blood calcium concentration to 4-6 mg%). By contrast, it was found that in pregnant cows with a chronic stimulation of parathyroid glandular activity secretion rates 50 to 100-fold greater than normal are seen with severe hypocalcemia. The rate of new hormone bio-synthesis necessary to sustain the rates of secretion in normal animals observed with severe hypocalcemia indicate that the gland content of hormone must be newly synthesized every few minutes. In the pregnant cow, the glands undergo hyperplasia. This results in sufficient adaptation of parathyroid glandular activity to support much greater rates of hormone secretion then are possible in the normal animal. By observing the rates of parathyroid hormone production during the spontaneous hypocalcemia that occurs with parturition and the onset of lactation in cows or inducing acute hypocalcemia in these animals through EDTA infusions it was found that parathyroid hormone secretion rates increased 50 to 100-fold above the normal rate in contrast to the 5 to 6 fold maximum increase detected in the normal animal. However, parathyroid hormone production still remained under proportional control by blood calcium. When parathyroid hormone concentrations were plotted against serum calcium concentrations in the chronically stimulated animals the same type of inversely proportional linear correlation was observed between calcium and hormone concentrations in blood. However, the slope of the line describing the relationship was as much as 6-fold higher than the slope of the relationship noted with acute stimulation of normal animals. It was evident that a finite time is required for this adaptation of parathyroid glandular activity to occur and for the hyperplastic glands to undergo involution once the period of chronic stimulation is passed. The latter was confirmed by giving EDTA infusions to animals some weeks after parturition; hypersecretory responses were still observed. The cause of this chronic stimulation in pregnant cows has not been definitely established but apparently the etiologic factor is the slow loss of calcium during pregnancy followed by a sudden loss with onset of lactation. These findings in pregnant cows concerning adaptation of the parathyroid glandular activity after chronic stimulation have served to evaluate states of secondary hyperparathyroidism in man (such as peudohyperparathyroidism and chronic renal disease).

3. Studies in human subjects have continued to further the application of the assay technique to the diagnosis of primary hyperparathyroidism and, more recently, to analyze the secondary abnormalities in parathyroid function that occur in such conditions as chronic renal disease. It has been possible to apply the immunoassay to the study of parathyroid disfunction in man despite the limitations of lower sensitivity (human parathyroid hormone differs immunologically from bovine parathyroid hormone).

Tests with the highly purified preparation of human parathyroid hormone have now established that some of the lower sensitivity seen with application of the bovine assay to measurement of human PTH is due to the weaker immunological reactivity of human hormone with the anti-bovine antiserum. By the use of the highly purified human preparation it has been possible to establish an immunoassay reference standard for human parathyroid hormone. This will permit all values for parathyroid hormone in human subjects to be expressed in absolute units rather than in bovine equivalent units and afford greater reliability.

243

Measurements by the assay confirm that production of excessive parathyroid hormone is involved in the syndrome of ectopic hyperparathyroidism. Excessive concentrations of parathyroid hormone in blood are found in the majority of patients with primary hyperparathyroidism that are tested. It has been possible to confirm that secretion of the hormone in these patients is indeed autonomous. Hypercalcemia induced with calcium infusion or hypocalcemia brought about by EDTA infusion does not result in any change in the rate of production of parathyroid hormone in these patients. By contrast, in patients with secondary hyperparathyroidism such as osteomalacia the increased rate of hormone secretion can still be suppressed by raising blood calcium. Examination of 30 to 40 patients with chronic renal disease has indicated that all patients have excessively high concentrations of hormone; in many cases hormone concentrations are higher than those found in primary hyperparathyroidism. Some of these patients may be suppressed with calcium infusion and extensive tests are now underway to determine how often parathyroid function can be suppressed in such patients and to relate this to their subsequent clinical course. Particular interest is centered around the changes in parathyroid activity that accompany the improvement of renal function with renal transplantation. The responses seen in patients with chronic renal disease and pseudohypoparathyroidism suggest that in analogy to the pregnant cows, a chronic stimulus (perhaps mild hypocalcemia) has been responsible for parathyroid glandular hyperplasia and adaptation to excessively high rates of parathyroid hormone production. However, in analogy with the situation in the pregnant cow parathyroid hormone production, at least in a majority of these patients, is still under the control of blood calcium concentration.

Significance to Heart Research: This work is part of the continuing effort of the laboratory to gain a greater understanding of the biosynthesis and secretion as well as the function of polypeptide hormones. The work is of considerable interest in elucidating the finely regulated control of hormone secretion, and, in turn, its regulation of calcium homeostasis, bone structure, and tissue metabolism in general.

Proposed Course of Research: It is hoped to extend the studies by the radioimmunoassay to a more detailed understanding of the control of parathyroid hormone secretion in states of both acute and chronic stimulation. It is evident that the assay is sufficiently sensitive for routine measurements in the variety of patients with disorders of parathyroid function and bone metabolism. Extension of these studies will constitute an important aid in understanding the role of parathyroid hormone in these disorders.

Publications:

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2. Deftos, L.J., Rabson, A.S., Buckle, R.M., Aurbach, G.D., and Potts, J.T.,Jr.: Parathyroid Hormone Production in Vitro by Human Parathyroid Cells Transformed by Simian Virus 40. Science 159: 435-436, 1968.

3. Potts, J.T., Jr., Buckle, R.M., Sherwood, L.M., Ramberg, C.F., Jr., Mayer, G.P., Kronfeld, D.S., Deftos, L.J., Care, A.D., and Aurbach, G.D.: Control of Secretion of Parathyroid Hormone. In, "Parathyroid Hormones and Thyrocalcitonin (Calcitonin)". Talmage, R.V. and Belanger, L.F. (eds) Excerpta Medica, p. 266-274, 1968.

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10. Sherwood, L.M., Mayer, G.P., Ramberg, C.F., Jr., Kronfeld, D.S., Aurbach, G.D., and Potts, J.T., Jr.: Regulation of Parathyroid Hormone Secretion: Proportional Calcium Lack of Effect of Phosphate. Endocrinology, (in press).

Serial No. NHI- 129
1. Laboratory of Molecular Diseases
2. Section on Polypeptide Hormones
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of the Structure of Parathyroid Hormone and Relationship of Structure to Function

Previous Serial Number: 312

Principal Investigators: John T. Potts, Jr., M.D.
Hugh D. Niall, M.D.
H. Bryan Brewer, Jr., M.D.
Leonard J. Deftos, M.D.
Henry T. Keutmann, M.D.

Other Investigators: Bess Dawson (Technical)
Rosemary Ronan (Technical)
Lynn Callahan (Technical)

Cooperating Units: NIAMD
Gerald Aurbach, M.D.
Bette Houston (Technical)
Jack Palmer (Technical)

Man Years:
Total: 4.5
Professional: 1.5
Other: 3

Project Description:

Objectives: To characterize the covalent structure and conformation of parathyroid hormone and the relationship of that structure to its biological and immunological function as well as its mechanism of action.

Methods Employed: The partially purified hormone is isolated by gel filtration on Sephadex G-100 followed by chromatography on carboxymethyl-cellulose. An overall yield of 70-80% is achieved; this estimate is based on the fractional recovery in the final product of the total bio-assayable and immunoassayable activity present in the crude gland extract. Purity of the hormone is assessed by polyacrylamide disc gel electrophoresis, thin layer chromatography, and amino-terminal end group determinations by the Edman procedure. The tryptic peptides of parathyroid hormone were prepared by digestion with the endopeptidase; other endopeptidases, exopeptidases, and various chemical reagents were also used to produce fragments and derivatives of the native polypeptide. Peptide fragments were 1) separated in two dimensions using paper electrophoresis and chromatography 2) fraction-

ated on columns of Sephadex G-50 and G-25 or 3) purified by ion exchange on Dowex-50. Amino acid composition of individual peptide fragments and derivatives was determined after acid or enzymic hydrolysis on the automatic amino acid analyzers, modified for high sensitivity. Detailed analysis of the composition of parathyroid hormone required development of several newer techniques of hydrolysis. Total enzymic hydrolysis was achieved in high yields using a procedure that employs incubation with papain and aminopeptidase M in the presence of 0.02 M mercaptoethanol. Acid hydrolysis in 1 to 2,000 v/v mercaptoethanol significantly improved recovery of methionine residues which proved useful in evaluating the methionine content of individual peptide fragments and in evaluating chemical derivatives of the hormone prepared by such techniques as cyanogen bromide cleavage. Bio-assays were performed in rats that had been parathyroidectomized after four days on a low calcium diet. Immunological activity was determined by the radioimmunoassay technique for parathyroid hormone.

Major Findings: Individual tryptic peptides of the native hormone were isolated and their amino acid composition determined. It was possible to tentatively align many of these peptides in their proper order by analysis of fragments of the native parathyroid hormone produced by the other chemical and enzymic methods.

With cleavage by dilute acid, sequential degradation with leucine aminopeptidase, and selective chemical modification of various aromatic and sulfur containing amino acid residues, it was learned that a minimum fragment at the amino-terminus of the molecule constituting only 35% of the total amino acid sequence of parathyroid hormone contained a structure requisite for both immunological and biological activity. Certain dissociations were evident between immunologic and biologic activity, but it was apparent that the same region of the molecule was important for both activities. The amino acid composition of much of the active fragment of the molecule was determined by analysis of the composition of various peptide fragments cleaved by trypsin and cyanogen bromide from the amino terminal portion of the molecule and by sequential Edman degradation (phenylthiohydantoin amino acids successively cleaved from the molecule were identified by gas-liquid chromatography).

Other studies have been aimed at isolation and characterization of human parathyroid hormone. A small amount of highly purified human hormone has been recovered following extraction and purification of 300 grams of human adenoma tissue. This has permitted amino acid analysis and physio-chemical characterization of the human hormone and detailed studies in the radioimmunoassay system thereby establishing conclusively that the human hormone although chemically similar differs significantly from the beef hormone in immunologic activity. These studies help to explain the problems with the sensitivity of the immunoassay for measurement in human plasma.

Significance to Heart Research: This work is part of the continuing effort of the laboratory to gain a greater understanding of the structure and function of protein molecules, particularly polypeptide hormones. The work, in general, is of a highly basic and theoretical nature, with obvious bearings on tissue metabolism in general and the control of calcium and

other ions.

Proposed Course of Research: It is hoped that the complete covalent structure of parathyroid hormone will be elucidated in studies over the next year or two. Accompanying this will be more detailed investigation of the regions of the molecule important for both biological and immunological activity. Among other efforts, it will be of particular interest to determine whether all derivatives retain both phosphaturic and calcium mobilizing activity.

Recent studies by other workers have indicated that effects of adenyl cyclase in kidney and bone that cause an increase in intra-cellular concentration of the 3', 5' cyclic AMP may be important in the mediation of the effects of parathyroid on kidney and bone. Even though a similar final mechanism may be involved in metabolic action it seems that the receptor sites, the plasma membrance in kidney or bone cells, may be sufficiently different in the two tissues that various structural derivatives of the molecule will bind more effectively to the cells of one organ rather than the other.

It is hoped to more fully appreciate the role of the conformation of parathyroid hormone in its biological activity by contrasting the specific biological activity of various derivatives in the whole animal bioassays, in in vitro preparations such as the perfused cat tiba, and more fundamental biochemical systems such as the adenyl cyclase assay system. A finding that a derivative of the hormone has biological activity more nearly equivalent to that of the native polypeptide when studied in the adenyl cyclase system but a much lower potency when the derivative is compared to the native polypeptide in the whole animal bioassay might point to the role of the entire amino acid sequence for stabilizing a specific conformation of the molecule which protects the hormone in plasma and aids delivery of intact hormone through the circulation to the receptor sites in kidney and bone.

Finally, it has become increasingly important to characterize the molecular sites of the molecule requisite for biological versus immunological activity. It is important to insure that peptide detected in the immunoassay system is biologically active. Preliminary reports from other workers have suggested that particularly in chronic renal disease there may be a significant concentration of immunologically reactive hormone with a much slower rate of disappearance from blood than the native polypeptide. This may be particulary prevalent in chronic renal disease where the damaged kidney tissue does not serve its usual role as a site for inactivation of the hormone. It is hoped that synthesis of the active fragment of the molecule can be undertaken and that analogs may be prepared which are more closely similar at least in immunological reactivity to the human hormone thereby bringing about improvements in the sensitivity of the immunoassay for detection of human parathyroid hormone in blood.

Publications:

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3. Aurbach, G.D. and Potts, J.T., Jr.: The Chemistry of Parathyroid Hormone Proceedings of the International Symposium on Drugs of Animal Origin. Leonardi, A and Walsh, J. (eds.) Milan, p. 167-171, 1967.

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7. Potts, J.T., Jr, Niall, H.D., Keutmann, H.T., Deftos, L.J., and Aurbach, G.D. Amino Acid Sequence and Structural Requirements for the Biological and Immunological Activity of PTH. Proceedings of the Third International Congress of Endocrinology, Excerpta Medica, (in press).

ANNUAL REPORT
LABORATORY OF CHEMICAL PHARMACOLOGY, NHI
JULY 1967 TO JUNE 1968

BIOLOGICAL CONTROL SYSTEMS

Adrenergic Neurochemical Transducer

Several years ago this laboratory conceived a theoretical model of the control system at sympathetic nerve endings which integrates processes that synthesize, store, inactivate, release and take up norepinephrine. This mode was called a "neurochemical transducer," because it translates electrical impulses into a precise quantity of free neurohormone which in turn acts on a target organ and releases mechanical or physical energy. Studies from this and other laboratories have indicated that norepinephrine (NE) is synthesized and stored in intracellular granules. Presumably, the NE in the granules is in equilibrium with a mobile pool which is separated from the receptor by the cellular membrane of the nerve ending. Nerve impulses depolarize the cellular membrane facing the receptor, thereby decreasing its permeability and allowing the norepinephrine to reach the receptor. Between each impulse, the membrane permeability is restored and the released NE recaptured by the action of an active transport system in the cellular membrane. NE in the transducer turns over rapidly, its level being controlled by a balance between synthesis and catabolism. For example, as reported last year, NE is metabolized at about 0.056 μg/g/hr in heart.

Drugs affect the transducer in diverse ways, such as altering the storage in the granules or the activity of the pump in the cellular membrane. When this laboratory first showed that reserpine impairs storage of biogenic amines, we believed that the drug caused amine depletion by blocking the pump. Other investigators later claimed that reserpine did not block the membrane but impaired the ability of granules to store the amines. During the past year the debate was resolved by studies on the metabolism of low concentrations of exogenous H^3-NE. Labeled NE was rapidly deaminated by heart slices from normal and reserpinized rats but was not deaminated by slices from immunosympathectomized animals. Thus, NE is not readily accessible to monoamine oxidase in non-neuronal tissue but is rapidly transported across the cellular membrane to the monoamine oxidase in sympathetic nerve endings in both normal and reserpinized hearts. Similar results were obtained by comparing the metabolism of H^3-NE by iris from normal and denervated rats.

These data suggest that uptake may be defined as that amount of H^3-NE which is deaminated plus the amount which is retained unchanged in the heart. Accordingly, perfusion studies revealed that hearts from animals pretreated with reserpine take up as much H^3-NE as do those from control animals. In contrast, desipramine and ouabain block uptake of H^3-NE by hearts from control or reserpine-treated rats, indicating that these substances block the transport of the amine.

It is possible that many of the drugs known to affect the adrenergic transducer may exert their action through more than one mechanism. Even though desipramine at a concentration of 2 μg/ml blocks NE uptake, it does not affect the half-life of H^3-NE in tissue slices and actually prolongs rather than shortens the half-life of H^3-NE in heart of living animals. Presumably, the prolongation is due to the enhancement of a negative feedback mechanism which controls NE synthesis, but the mechanism is obscure. In contrast, at high concentrations (25-50 μg/ml) desipramine markedly decreases the half-life of H^3-NE in tissue slices. Moreover, when BW392C60, a sympatholytic drug, is injected into brain ventricles, it slows the release of H^3-NE not only in control rats but also in reserpine-pretreated rats. Thus, it acts both by preventing release of NE and by inhibiting monoamine oxidase.

EFFECTS OF CATIONS ON UPTAKE, STORAGE AND RELEASE OF NOREPINEPHRINE AND SEROTONIN

Previous work from this laboratory has shown that Na^+ is required for the uptake of H^3-NE into heart slices. The uptake is facilitated by low concentrations (3-26 mM) of K^+ but antagonized by higher concentrations (>26 mM). Accordingly, the half-life of H^3-NE in heart slices from rats pretreated with the radiolabeled amine was shorter in medium containing no Na^+ or high concentrations of K^+ than it was in Krebs-bicarbonate solution. Similar findings have been obtained with synaptosomes, which are pinched-off nerve endings isolated from brain homogenates.

The similarity in the effects of Na^+ and K^+ on uptake of serotonin and NE, and their effects on uptake of certain amino acids and sugars, suggests a common general mechanism for active transport. Based on the concepts of Crane et al. and Kipnis and Parrish, the following mechanism has been proposed as a working model:

1) On the outer surface of the plasma membrane, bathed in a medium containing high $[Na^+]$ and low $[K^+]$, the Na^+ reacts with the carrier to form a complex which has a high affinity for the amine.

2) The Na-carrier-amine complex is carried to the inner surface of the membrane, where the relative concentrations of the monovalent ions are reversed. The complex dissociates and the carrier is converted to a K^+-carrier complex, which is carried to the external surface of the membrane.

3) The intracellular Na^+ and the extracellular K^+ are then transported by the Na^+-K^+-dependent ATPase, that is the Na^+ pump, which maintains the Na^+ and K^+ gradients.

Obviously this model is an oversimplification for it does not account for the finding, reported last year, that in a medium free of Na^+ and K^+ NE is more rapidly depleted in the presence of Ca^{++} than in the absence of Ca^{++}, nor does it account for the finding that at low concentrations desipramine blocks uptake of NE but does not enhance release. Perhaps efflux of NE is also mediated by the carrier or perhaps Ca^{++} and desipramine play roles in the storage mechanism in the granules.

The model suggests that the Na^+-K^+-ATPase plays an indirect role in the carrier mechanism. In accord with this view, ouabain, which rapidly inhibits Na^+-K^+-ATPase, blocks NE uptake only after it has been preincubated with the tissue for at least 7 to 15 minutes. Curiously, ouabain has only a slight releasing action. In hearts perfused with ouabain, the $[K^+]$ was decreased to about one half of its normal value, and the $[Na^+]$ was tripled at a time corresponding to the onset of inhibition of NE uptake.

Alterations in the ionic milieu may also alter NE metabolism in vivo. Treatment of rats with Li^+ in daily doses of 3 mmol/kg for 10 days decreased the half-life of H^3-NE in brain from about 6 hours to about 2 hours.

Sodium and Potassium Dependent ATPase

Current studies on the mechanism of action of an enzyme preparation from nervous tissue confirm that sodium activates an intermediate phosphorylation of the enzyme by its energy-providing substrate, ATP, but suggest that the phosphorylated form which actually takes part in the reaction may not be the generally postulated acyl phosphate bond. Whatever the intimate site of phosphorylation may be, the radioactively labeled phosphoprotein has helped to resolve a problem which has long hindered the purification of membrane-bound enzymes. Thus, the catalytic proteins of ATPase appear to be functional only as they are associated in an insoluble matrix which derives from the membrane and which contains a number of extraneous proteins. Labeling of the protein with radioactive ATP and sodium followed by dissociation of the hydrophobic bonding of the matrix and electrophoretic separation of the products have led to the isolation of a single protein which, although inactive when disembedded, is clearly a component of the transport ATPase. Other studies indicate that levels of this same protein increase as transport ATPase activity appears during the development of rat brain and kidney.

Work still in progress indicates that certain inhibitors will offer insight into ATPase mechanisms. Thus, aliphatic substitutions on hydroxylamines appear to stabilize the membrane against the otherwise inhibitory effects of these compounds. Kinetic studies of the effects of oligomycin suggest that interaction with the enzyme occurs only after formation of an active complex including ATP and sodium. The macrolide antibiotics may thus offer a sensitive test for conformational changes.

SYMPATHETIC TARGET SITES

α-Receptors

Because of the importance of noradrenergic systems in cardiovascular control, an attempt is being made to label the alpha-noradrenergic receptor by covalently attaching a radioactive inhibitor and to use the radioactivity as a guide for isolation and identification of receptor components. Studies to date suggest that the alpha-receptor may be more complicated than commonly supposed. The irreversible inhibitors used for labeling may alkylate sites adjacent to but not identical with the receptor proper. The studies also indicate that occupation of these sites alters the affinity of the receptor for the natural transmitter, NE.

NE Synthesis in Adipose Tissue

Since the release of free fatty acids (FFA) from adipose tissue is thought to be controlled by the sympathetic system, it was of interest to study the rate of synthesis in brown (interscapular) and white (epididymal) adipose tissue. As calculated from the rate of disappearance of NE after inhibition of catecholamine synthesis by α-methyl-tyrosine, the basal rate of NE synthesis in brown fat is faster than in heart, whereas synthesis in white fat is about 1/5 that in heart. The rate of NE synthesis was markedly increased by acute and chronic cold exposure, muscular exercise and stress by restraint, all of which increase plasma levels of FFA. In contrast, NE synthesis was not affected by fasting even though fasting increases FFA in plasma.

In rats amphetamine, which also elevates plasma FFA levels, depletes NE stores in interscapular and epididymal fat pads more rapidly than it depletes NE stores in heart. Heart NE levels remained unchanged during the first few hours after amphetamine administration and then declined to about 50% of controls at 24 hours. Interscapular NE levels declined to about 60% of control levels during the first hour and returned to normal levels at 24 hours. Epididymal NE levels decline to 25% of the control levels at 6 hours and then returned to normal at 24 hours. In contrast, cocaine failed to deplete NE in heart and adipose tissues, and metaraminol depleted NE in interscapular and epididymal fat pads and heart at the same rate.

Effect of Cortisone

Treatment of rats with cortisone enhances the lipolytic effects of norepinephrine on adipose tissue. Intensive cortisone treatment, however, enhances neither adenyl cyclase activity nor the NE-stimulated rise in cyclic AMP levels in epididymal fat pads. Preliminary experiments suggest that cortisone pretreatment causes an increase in the total amount of lipase.

ROLE OF CYCLIC AMP IN BRAIN

Dibutyryl 3',5'-AMP

As reported last year brain contains large amounts of adenyl cyclase, which is apparently localized in the nerve endings. The distribution of the enzyme, however, does not parallel the distribution of NE,5-HT or phosphodiesterase. During the past year it was found that the 3',5'-AMP levels were unusually high in cerebellum, a part of the brain which contains little phosphodiesterase.

To establish a possible role for 3',5'-AMP in brain, dibutyryl, 3',5'-AMP was injected into different brain areas and behavioral changes were observed. Injection of the compound into the right ventricle of rat brain produced intense hyperactivity followed by convulsions. Pretreatment of the rats with chlorpromazine or reserpine enhanced the hyperactivity rather than prevented it. A similar hyperactivity was observed after dibutyryl 3',5'-AMP was injected into the intracisternal area of cats and rabbits and into the hypothalamus of rats.

353

Dibutyryl 3',5'-AMP, injected into the hypothalamus of cats in doses of 100 μg, caused intense ergotropic stimulation characterized by hyperactivity, sham rage, mydriasis, salivation, hypothermia and hallucinatory behavior. Injection of 500 μg of the compound caused convulsions and lethal hyperthermia. Injection of 100 μg of the compound into the reticular formation caused a marked catatonia, which lasted for several hours. Since none of these effects is produced by injecting either 5'-AMP or dibutyryl 5'-AMP, these observations suggest that 3',5'-AMP may play an important role in brain function. In accord with this view, other workers have shown that electrical stimulation increases the level of 3',5'-AMP in the various brain areas and causes responses similar to those described above.

In this connection it is noteworthy that caffeine and theophylline, which are known to block phosphodiesterase in adipose tissue, potentiate the central stimulant action of amphetamine as measured by confinement motor activity (CMA). In contrast, the stimulant action of caffeine is reduced by pretreating rats with the alpha-adrenergic blocking drugs, phentolamine, phenoxybenzamine and chlorpromazine, and by the beta-adrenergic blocking drug, propranolol. Whether these effects are related to alterations in the brain level of 3',5'-AMP, however, remains to be determined.

Cyclic AMP in Arterial Tissues

Evidence from other laboratories suggests that adrenergic stimulation increases 3',5'-AMP levels in organs containing beta-receptors and decreases 3',5'-AMP levels in organs containing alpha-receptors. In this laboratory attempts to relate the effects of epinephrine perfusion on blood pressure with changes in 3',5'-AMP levels in arterial tissue suggest that epinephrine causes only a small and possibly nonsignificant decrease in 3',5'-AMP levels in carotid, mesenteric and femoral arteries.

THE PHYSIOLOGIC ROLE OF NONMAST CELL HISTAMINE

This laboratory has previously reported that nonmast cell histamine is located throughout the body tissues and is resistant to the depleting effect of compound 48/80. The apparent synthesis of nonmast cell histamine has been evaluated by labeling the pool of histamine and measuring the decline of the radiolabeled amine under steady-state conditions. Such studies revealed that the apparent synthesis of nonmast cell histamine in various glandular tissues was related to the functional state of the organ. In addition, studies on the conversion of H^3-histidine to H^3-histamine as measured by benzene sulfonyl derivative technique indicated that histidine decarboxylase was present in tissues containing nonmast cell histamine. During the past year, studies were carried out in an attempt to corroborate these results.

Perfusion of isolated submaxillary glands with blood containing either H^3-histidine or C^{14}-O_2-H-L-histidine failed to demonstrate the presence of histidine decarboxylase in this tissue. The small amount of $C^{14}O_2$ formed in the system was identical to that formed in blood and was not increased by stimulation of the submaxillary gland. This study showed, however, that submaxillary glands convert H^3-histidine to a product which interferes with the assay of labeled histamine by the benzene sulfonyl derivative technique. The

product, tentatively identified as urocanic acid, was also present in saliva. Since histidine decarboxylase is apparently not present, the source of histamine in the submaxillary must be the blood even though the histamine level in plasma is usually undetectable.

It was shown that histamine is synthesized in leucocytes from blood of cats and rats. It is possible that the rate-limiting step in histamine synthesis is not the histidine decarboxylase but the transport of histidine into the tissue, for histamine formation as measured by $C^{14}O_2$ release was inhibited by substances, such as ouabain, methionine and iodoacetate, which are known to inhibit amino acid transport systems. Similarly, the transport of histidine into rat gastric mucosa may be the rate-limiting step of histamine synthesis in this organ, for methionine blocked the uptake of H^3-histidine and the formation of H^3-histamine by slices of gastric mucosa, but did not inhibit histamine formation by purified histidine decarboxylase. It was also shown that transport of L-histidine into gastric mucosa was lower in fasted than in fed rats. These findings could possibly account for the effects of fasting on histamine synthesis, though fasting is also known to decrease the activity of histidine decarboxylase in homogenates of gastric mucosa.

Effects of Gastrin on Histamine Levels and Gastric Secretion

Administration of gastrin to rats decreases the level of histamine in gastric mucosa and then causes an increase in histidine decarboxylase activity which leads to replenishment of the histamine store. A second dose of gastrin caused no further depletion of histamine but still evoked an increas in output of acid. Thus, the histamine levels in gastric mucosa after gastrin administration are still sufficiently high to mediate gastrin-induced acid formation.

Mechanism of Histamine Release by Reserpine

In contrast to 5-HT and NE, which are stored in neuronal vesicles, histamine is localized in the cell sap of gastric mucosal cells. The finding that reserpine released 5-HT and NE by acting on the vesicles in neurons and not by inhibiting amine transport by the cellular membrane thus prompted studies on the mechanism by which reserpine releases histamine from gastric mucosa. These studies revealed that reserpine inhibits histidine decarboxylase in gastric mucosa in rats subjected to bilateral gastric vagotomy as well as in unoperated rats. The inhibitory effect is maximal within one hour and persists for at least five hours. Histamine levels decline to their minimum values between 2 to 5 hours after reserpine administration. Thus, the decrease in histamine levels caused by reserpine may be partially due to blockade of histidine decarboxylase which is not related to stimulation of the parasympathetic system.

DRUG METABOLISM

Plasma Levels of Chlorpromazine in Man

Last year this laboratory in collaboration with Dr. Marshall at St. Elizabeth's Hospital reported large variations in the plasma levels of

chlorpromazine in patients receiving the drug chronically. Studies during the past year have revealed that the variability may be due to differences in absorption of the drug. Whereas the volume of distribution of chlorpromazine after intramuscular injection is approximately 7 times the body weight, the volume of distribution after oral administration varies from 7 to 70 times the body weight. The biological half-lives of the drug varied from 1.9 to 3.0 hours, although in 80% of the patients the half-life was 6 hours or less. Preliminary studies have also shown that pretreatment of patients with phenobarbital enhances the rate of chlorpromazine elimination, thereby decreasing the plasma level.

Experiments in rats revealed that the biological half-life of chlorpromazine in brain, liver and muscle are similar to the biological half-life in plasma, suggesting that equilibrium among the major compartments had been established. Thus, measurement of the plasma levels provides a reasonable estimate of the levels in other tissues.

Owing to the nature of schizophrenia, it has not been possible to obtain an exact relationship between the plasma level of the drug and the response. Nevertheless, the peak plasma levels of chlorpromazine in patients who responded satisfactorily were 50-500 ng/ml. Patients with lower plasma levels were generally unresponsive to the drug.

Drug Metabolism in Identical and Nonidentical Twins

Studies from this and other laboratories have demonstrated marked individual variations in drug metabolism in man. Since prior treatment of patients with drugs is known to alter the rate of drug metabolism, these individual variations in drug metabolism may arise from either environmental or genetic causes. Studies on the metabolism of phenylbutazone, antipyrine and dicoumarol in man revealed that there are greater intra-pair differences in drug metabolism in nonidentical twins than in identical twins. These findings suggest that the major variation in drug metabolism in healthy individuals is due to genetic rather than environmental factors.

Enzymatic Mechanism of Drug Metabolism

During the past few years, it has become evident that most lipid-soluble drugs are oxidized in liver microsomes by NADPH-dependent enzyme systems which include cytochrome P-450. The finding that substrates and inhibitors of these enzyme systems altered the absorption spectrum of the cytochrome, even in the absence of NADPH, suggested that combination of the substrate with the enzyme might be the initial step of the reaction. Thus,

$$\text{Substrate} + \text{Cytochrome P-450}_{(ox)} \rightleftharpoons \text{Substrate - Cytochrome P-450}_{(ox)}$$

$$\text{Substrate - Cytochrome P-450}_{(ox)} + \text{NADPH} \xrightarrow{\text{cytochrome c reductase}} \text{Substrate - Cytochrome P-450}_{(red)} + \text{NADP}$$

$$\text{Substrate - Cytochrome P-450}_{(red)} + O_2 \rightleftharpoons \text{Substrate - Cytochrome P-450 - } O_2$$

$$\text{Substrate - Cytochrome P-450} \longrightarrow O_2 \text{ - Cytochrome P-450}_{(ox)} + \text{Oxidized Substrate}$$

Evidence obtained during the past year indicates that the reduction of the substrate - cytochrome P-450 complex may be the rate-limiting step. Accordingly, the N-demethylation of ethylmorphine by liver microsomes from various species was more closely related to the rate of cytochrome P-450 reduction than to the amount of cytochrome P-450, the magnitude of the spectral change induced by ethylmorphine, or the activity of NADPH cytochrome c reductase. Similarly, differences in the rate of N-demethylation of ethylmorphine by smooth and rough surfaced microsomes from rabbit liver closely paralleled differences in the rate of cytochrome P-450 reduction.

Addition of substrates and inhibitors altered the rate of cytochrome P-450 reduction. Those substances which cause type I spectral changes stimulated reduction of the cytochrome, whereas those which caused type II spectral changes slowed its reduction. Differences in the magnitude of the ethylmorphine-induced stimulation in liver microsomes from male and female rats paralleled the sex difference in the metabolism of this substrate.

The activity of the enzyme system may be impaired in various ways. Pretreatment of animals with carbon tetrachloride decreases enzyme activity by destroying cytochrome P-450 as measured by decreases in the amount of heme in liver microsomes as well as by decreases in the absorbancy at 450 mμ. The effects of carbon tetrachloride on the microsome enzymes may be delayed by pretreatment of the animals with SKF-525A but not by pretreatment with antioxidants, which prevent CCl_4-induced fatty liver, or by potent inhibitors of drug metabolism, such as Lilly 18947 or its primary amine derivatives, DPEA.

Adrenalectomy of male rats impairs the enzyme system by decreasing the amount of NADPH cytochrome c reductase. Treatment of adrenalectomized animals with cortisone restores the activity of NADPH cytochrome c reductase and ethylmorphine N-demethylase.

N-Hydroxylation

Since carbon monoxide does not inhibit the N-hydroxylation of primary and secondary amines, it is possible that this reaction is catalyzed by an enzyme which does not involve cytochrome P-450. Accordingly, marsilid blocks the p-hydroxylation of aniline but does not alter its N-hydroxylation. Moreover, the K_m values for the two reactions differ.

GENERAL PHARMACOLOGY

Effects of Reserpine in the Submaxillary Gland

The administration of reserpine to rats changes the structure of a protein in submaxillary gland. No change is caused by direct action of reserpine on the protein. The structural change, although insufficient to alter the chromatographic behavior of the protein, dramatically changes its surface properties as manifested by dye adsorption and by aggregation with other proteins. In collaborative studies with the National Institute for Arthritis and Metabolic Diseases, reserpine has been found to reduce the fluxes of calcium between blood plasma and the submaxillary gland. Temporal relationships make it unlikely, although not impossible, that alterations in

submaxillary calcium induce the changes in protein. Since reserpine induces long-lasting changes in the properties of the membranes of nerve endings, it is hoped that the newly isolated protein can be demonstrated to play a role in membrane function.

Enzymatic Mechanism of Mammalian Histidine Decarboxylase and Histamines

It has been postulated that formation of a double bond in the side chain of histidine and histamine may occur as an intermediary step in the decarboxylation of histidine and the deamination of histamine. The availability of L-histidine labeled in the side chain with H^3 ($R\text{-}CH^3H\text{-}CH(CO_2H)\text{-}NH_2$) permitted us to determine whether a double bond was formed during its decarboxylation ($R\text{-}CH = C(CO_2H)\text{-}N$ = Pyridoxal). Since H^3 should be released if a double bond were formed, the finding that no ^3H_2O was formed during the conversion of H^3-histidine to histamine by mammalian histidine precludes the formation of the proposed intermediate. In contrast, incubation of histamine similarly labeled in the side chain with H^3 ($R\text{-}CH^3H\text{-}CH_2\text{-}NH_2$) with mammalian histaminase led to the formation of ^3H_2O, suggesting that formation of a double bond is an intermediate step in the action of histaminase. Since the release of H^3 was quantitative, it is possible that this reaction may be utilized in the development of a specific assay for histaminase in tissues.

Rates of LDH 5 Synthesis

The relative proportions of the isozymes, LDH 5 and LDH 1, vary markedly from one tissue to another. Preliminary experiments indicate that in heart, which contains small amounts of LDH 5, the enzyme has a half-life of about 2 days, whereas in skeletal muscle, which contains large amounts of LDH 5, the enzyme has a half-life of about 40 days. These studies suggest that the tissue specific isozyme patterns are caused largely by tissue differences in the turnover of individual isozymes.

Blockade of Pentylenetetrazol Convulsion in Mice

Convulsions produced by pentylenetetrazol were prevented by pretreating mice with chlorisondamine, bretylium, BW392C60 and propranolol. The anticonvulsant effect of chlorisondamine was reversed by injection of either epinephrine or isoproterenol. Moreover, the specific EEG pattern of pentylenetetrazol was not altered by pretreatment of mice with chlorisondamine. Thus, the anticonvulsant effect is probably mediated by blockade of the peripheral sympathetic nervous system. Since these ganglionic and adrenergic blocking agents also prevent the increase in plasma FFA and glucose, the anticonvulsant activity may be related to lipid and glucose metabolism.

Toxic Effects of Phenylbutazone

Phenylbutazone administered to rats in doses 100 to 200 mg/kg decreased the ability of liver microsomes to incorporate amino acids. The inhibition of amino acid incorporation also occurred when 0.5 mM phenylbutazone was incubated with liver slices or when 2-4 mM phenylbutazone was incubated with microsomes. Although the mechanism of inhibition by phenylbutazone is unclear, it may be related to the uncoupling effect of the drug on oxidative phosphorylation.

Cyclic AMP

A method has been developed for the assay of 3',5'-AMP in pmole amounts. In this method 3',5'-AMP is converted to a trimethylsilyl derivative and separated from the trimethylsilyl derivatives of other mononucleotides by GLC. With an argon ionization detector, as little as 150 pmoles of 3',5'-AMP may be assayed. But with a phosphorus detector, 60 pmoles are measured. The method may also be used for the assay of 3',5'-GMP and 3',5'-IMP.

Assay for Aminoguanidine

A sensitive method based on the reaction of p-nitrobenzaldehyde with aminoguanidine has been developed. The method has been used to determine the distribution of this histaminase inhibitor in animals.

Serial No. NHI- 130
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effect of Sympathetic Denervation on the Uptake and Metabolism of NE by Iris Tissue

Previous Project Number: None

Principal Investigators: Dr. Peter Keen
Dr. Frederick Leitz
Dr. Donald F. Bogdanski

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.3
Professional:	0.3
Other:	0.0

Project Description:

Objectives: To determine uptake and metabolism of tritiated norepinephrine (H^3-NE) by sympathetically denervated tissues in comparison with normal controls. The object is to determine whether amines released by reserpine are metabolized intraneuronally or extraneuronally.

Methods Employed: Denervated irides of rabbits were prepared by chronic superior cervical gandiectomy.

Major Findings: 1) H^3-NE was metabolized by monoamine oxidase in control irides but not by that in denervated irides. Since neurons are required for the metabolism it must be concluded that the amine is metabolized mainly by intraneuronal monoamine oxidase. Furthermore, the amine must be transported into the neuron.

2) Reserpine (5 mg/kg i.p.) did not prevent metabolism in normal tissues when given 4 hours before the experiment. The preliminary data indicate that reserpine did not block the uptake of H^3-NE into the sympathetic neurons.

3) Desmethylimipramine (DMI) prevented metabolism in normal and reserpinized irides indicating that DMI blocked the uptake of H^3-NE into the neurons.

Significance to Bio-medical Research and the Program of the Institute: These studies help to define the mechanism of action of important clinically useful drugs.

Proposed Course of Project: The project, as such, is terminated. However, the information gained will be useful in the design and interpretation of other experiments relating to the biochemical transducer at the nerve ending.

Honors and Awards: None

Publications: None

361

Serial No. - NHI-131
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Site of Action of Reserpine

Previous Serial Number: None

Principal Investigators: Dr. F. Leitz, and Dr. F. Stefano

Other Investigators: None

Cooperating Units: During the first 4 months Dr. Stefano was a fellow from Consejo Nacional de Investigaciones Cientificas y tecnicas, Argentine Republic

Man Years:

Total:	0.25
Professional:	0.25
Other:	0

Project Description:

Objectives: When this laboratory first showed that reserpine impairs the storage of biogenic amines we believed that the drug caused amine depletion by blocking the membrane pump. Later other investigators claimed that reserpine did not block the membrane pump but impaired the ability of the storage granules to store the amines. According to the former theory, NE is released from the nerve membrane onto MAO in surrounding tissues. According to the second theory NE is released from granules onto MAO in nerve endings. This theory holds that the membrane pump continues to function in the presence of reserpine, but that in the absence of storage capability, the NE is rapidly metabolized.

The purpose of the present study is to test which of the above hypotheses is correct.

Methods Employed: Rat heart slices from control, reserpinized and immunosympathectomized animals were incubated with H^3-NE. Isolated rat hearts from reserpinized and normal animals were isolated and perfused by the Langendorf technique with various concentrations of H^3-NE for 10 min. The perfusates were collected over acid. The tissue homogenates and the perfusates were adjusted to pH 6.5 and passed through a Dowex-50 column. NE was retained in the column while the acid metabolites passed through. The NE was then washed off the column with 2 N HCl. Tritium in various samples was determined by standard procedures.

362

Major Findings: Labeled NE was rapidly deaminated upon incubation with heart slices from normal and reserpinized rats. In contrast, H^3-NE incubated with heart slices of immunosympathectomized rats was not metabolized. The activity of MAO measured in homogenates was found to be similar in all three preparations. These results suggested that NE is not readily accessible to MAO in muscle cells but readily penetrates sympathetic nerve endings in both normal and reserpinized hearts where it is metabolized by MAO.

The studies were expanded using the isolated rat heart. Uptake was define as the H^3-NE taken up by nerve endings regardless of whether it accumulated in the heart or is metabolized by MAO and is found in the perfusate as deaminated products.

Our results show that perfusion fluid from the hearts of control animals contains negligible amounts of metabolites. Approximately 95% of the H^3-NE taken up by the heart is retained by the heart as unchanged amine. In the heart from reserpinized rats the H^3-NE retained by heart is only 5-10% of that retained by control rats. Analysis of the perfusate from the reserpinized heart showed the presence of acid metabolites equivalent to about 95% of the H^3-NE accumulated by the normal heart. Thus according to the new definition of uptake (NE retained plus NE metabolized), the reserpinized heart takes up NE as readily as does the normal heart. The experiments were done with the following concentrations of NE: 2, 10, 25, 50, and 75 ng/ml.

To confirm that this metabolism actually occurrs in sympathetic neurons DMI and ouabain were used as specific blockers of NE uptake. In control and reserpinized heart perfused with DMI and H^3-NE, or with ouabain and H^3-NE, the amine but not its metabolites was found in the perfusates. These drugs block NE uptake into the normals by 95%. Thus normal and reserpinized hearts in the presence of DMI and ouabain, behave similarly: there is no NE accumulation in the heart and no H^3-NE metabolite in the perfusate.

Significance to Bio-medical Research and the Program of the Institute: These results strongly suggest that the membrane pump is not affected by reserpine treatment hence the drug acts on granules. Furthermore, most of the MAO in heart is present in non-neuronal tissue and is not accessible to exogenous NE.

Proposed Course of Project: It is proposed to use the new definition of uptake (uptake equal accumulation plus metabolism) as a tool to follow the course of reserpine action. In addition we propose to look into the possible role of MAO in non-neuronal tissue.

Honors and Awards: None

Publications: None

363

Series No.- NHI-132
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Comparison of the Uptake and Storage of Exogenous H^3-NE by Heart Slices, the Isolated Heart, and the Whole Animal

Previous Serial Number: None

Principal Investigators: Dr. F. H. Leitz, and Dr. F. Stefano

Other Investigators: None

Cooperating Units: During the first 4 months Dr. Stefano was a fellow from Consejo Nacional de Investigaciones Cientificas y Tecnicas, Argentine Republic

Man Years:

Total:	0.25
Professional:	0.25
Other:	0

Project Description:

Objectives: The uptake and disappearance of H^3-NE by sympathetic neurons was compared in rat ventricle slices and the isolated perfused rat heart.

Methods Employed: Rat ventricle slices were incubated for 1 hr in Krebs bicarbonate solution containing 2 ng/ml H^3-NE. The slices were then transferred to media containing no NE, and the H^3-NE efflux measured for 1-3 hr. Isolated hearts were perfused with H^3-NE (2 ng/ml) for 30 min, and then for 90 min with NE-free Krebs solution. Rates of efflux were measured by sampling the bath at various times. Standard procedures were used for counting the amount of H^3 present in the baths.

Major Findings: When slices obtained from control animals were loaded with H^3-NE *in vitro* and then transferred to NE-free media, the H^3-NE thus acquired was not retained, but disappeared at a rate corresponding to a half-life of 1-2 hr. In contrast, the half-life of H^3-NE disappearance from heart slices from rats given the labeled amine *in vivo* 18 hr previously was 10 hr. Thus uptake and storage by heart slices *in vitro* is quite different to uptake and storage *in vivo* and should not be used for the study of drug action. To determine if a slow equilibrium between free (labeled) and bound (unlabeled) NE could explain the *in vitro* results, slices were incubated for 3 hr with H^3-NE in media 199 (a more complete media) before transfer to NE-free media.

364

Again a rapid efflux of the H^3-NE resulted. The results could not be explained by a more rapid metabolism in the slices since more than 95% of the tritium was present as unchanged H^3-NE. Experiments with isolated atria produced similar results.

When isolated hearts were perfused first with H^3-NE and then with NE-free Krebs solution for 90 min, most of the H^3-NE was retained in the heart. The half-life of efflux was similar to that *in vivo*. The isolated heart appears to be a satisfactory system for studying *in vitro* NE uptake.

Significance to Bio-medical Research and the Program of the Institute: These studies indicate that many reports equating uptake and storage by slices with uptake and storage *in vivo* may be in error. Our results suggest that uptake in slices involves a membrane function only and that the NE is not taken up by storage granules.

Proposed Course of Project: Similar studies with NE and other amines will be carried out in other tissues and the isolated heart with a view to studying the action of drugs in sympathetic neurons.

Honors and Awards: None

Publications: None

365

Serial No.- NHI-133
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Paradoxical Release of NE by DMI

Previous Serial Number: None

Principal Investigators: Dr. F. J. E. Stefano and Dr. F. H. Leitz

Other Investigators: None

Cooperating Units: During the first 4 months Dr. Stefano was a fellow from Consejo Nacional de Investigaciones Cientificas y tecnicas, Argentine Republic

Man Years:

Total:	0.25
Professional:	0.25
Other:	0.0

Project Description:

Objectives: Previous reports from this laboratory indicate that DMI and other tricyclic antidepressants block the uptake of NE into sympathetic neurons by an action on the neuronal membrane, and slows down the spontaneous efflux (turnover rate) of endogenous NE from the neurones by an action that appears to be in part on the granule membrane. Other reports have suggested large concentrations of these drugs actually release NE from nerve endings as well as 5HT from platelets. We proposed to investigate this problem using *in vivo* labeled heart slices.

Methods Employed: Ventricle slices prepared from rats given 100 µc H^3-NE/kg 18 hr previously were preincubated for 1 hr in Krebs bicarbonate solution then transferred to Krebs solution containing various amounts of DMI. The rate of H^3-NE efflux was measured by sampling the bath at various times. Rat hearts were isolated and perfused by the Langendorf technique with Krebs-bicarbonate solution.

Major Findings: When heart slices were incubated in solutions containing 1 µg/ml DMI or less, the H^3-NE efflux rate was similar to that from control slices ($T^1/_2$ about 10 hr). DMI concentrations of 25 µg/ml decreased the half-life of NE to 4 hrs, while 50 µg/ml decreased the half-life to 1 hr. This rate is similar to the maximum rates of release achieved by metaraminol, guanethidine, and tyramine. Isolated hearts were perfused with tracer doses of H^3-NE (2 ng/ml) for 15 min and then perfused with DMI (125 µg/ml). The

perfusates were adjusted to pH 8.4, the H^3-NE absorbed onto alumina and eluted with 0.2 N acetic acid. About 80% of the released H^3 in the perfusate was accounted for as NE.

Significance to Bio-medical Research and the Program of the Institute: The paradoxical effects of large levels of DMI might yield some insight into the action of antidepressant drugs with cellular membranes and increase our understanding of the processes by which NE is accumulated, stored and released.

Proposed Course of Project: Attempts will be made to show whether DMI releases NE by an action on the neuronal membrane or on the granules. Advantag will be taken of recent techniques in which stable free radicals are attached to protein membranes. Conformational changes produced by drugs are determined by changes in the electron spin of the free radical. Similar experiments will be carried out with synaptosomes.

Honors and Awards: None

Publications: None

Serial No. - NHI-134
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of Action of Ouabain on the Blockaded Uptake of NE into the Sympathetic Neurons

Previous Serial Number: None

Principal Investigator: Dr. F. H. Leitz and Dr. F. J. E. Stefano

Other Investigators: None

Cooperating Units: During the first 4 months Dr. Stefano was a fellow from Consejo Nacional de Investigaciones Cientificas y Tecnicas, Argentine Republic

Man Years:

Total:	1.0
Professional:	1.0
Other:	0.0

Project Description:

Objectives: A number of reports indicate that ouabain inhibits NE uptake by sympathetic neuron. The precise mechanism of this action is unknown, but it is thought to be different from that of membrane blockers such as cocaine and DMI. This report describes the action of ouabain on NE uptake in the isolated perfused guinea pig heart.

Methods Employed: The hearts of male guinea pigs (400-500 g) were isolated and perfused according to standard Langendorf technique. Krebs-bicarbonate solution was used as the perfusion medium. The hearts were perfused with H^3-NE (2 ng/ml) and the amine uptake measured by assay of tritium in the heart. In the determination of intracellular [Na^+] and [K^+], extracellular space was measured by C^{14} inulin and total water content was determined by drying heart tissue to constant weight. Standard procedures were used for counting H^3 and C^{14} activity.

Major Findings: H^3-NE uptake was measured for periods of 4, 10, and 20 min. The rate of NE uptake was linear for 20 mins. When ouabain (1×10^{-4} M) was present together with H^3-NE, the uptake was unchanged after 4 min, reduced by 40% after 10 min, and by 50% after 20 min. In the presence of DMI (1 µg/ml), the H^3-NE uptake was blocked by 90% at all time points.

The time required for ouabain to block H^3-NE uptake, was determined by perfusing hearts with ouabain (1×10^{-4} M) for various times before a 4 min perfusion of H^3-NE plus ouabain. These studies show that ouabain does not

affect the uptake of H^3-NE for about 6 min (2 min pretreatment and 4 min H^3-NE plus ouabain). After 7 min perfusion (3 min pretreatment and 4 min H^3-NE plus ouabain) a slight inhibition (20%) resulted. Inhibition progressively increases with time reaching a maximum of about 90% after 15 min ouabain pretreatment.

The effect of various concentrations of ouabain on NE uptake was determined after the 15 min pretreatment period. At a ouabain concentration of 5×10^{-5} M or higher, inhibition was 80% or more, at 5×10^{-6} M, inhibition was 60%, and at 1×10^{-6} M, inhibition was essentially zero.

To determine if ouabain released NE in addition to blocking its uptake, we perfused a heart, [previously loaded (*in vitro*) with H^3-NE] with ouabain (1×10^{-4} M) for two hr. During the first 40 min, efflux of H^3-NE was the same in control and ouabain treated hearts. After 40 min perfusion with ouabain the efflux of H^3-NE was somewhat faster. After 2 hr the control heart retained 83% and the ouabain heart 65% of its original H^3-NE content.

The intracellular concentrations of Na^+ and K^+ were related to the uptake of H^3-NE after perfusion with ouabain (1×10^{-4} M). At 4 min, Na^+ and K^+ values in control and ouabain hearts were similar, although $[Na^+]$ was increased 2-fold and $[K^+]$ was reduced by about 12%. At 7 min, $[Na^+]$ had continued to increase (2.2 times normal) and $[K^+]$ had declined by 50%. At 20 min, the $[Na^+]$ was 3 times normal and the $[K^+]$ was reduced by 55%. In perfused controls, after 20 min, $[Na^+]$ was 2 times normal and $[K^+]$ was lower by 25%. In contrast, DMI (1 µg/ml) had no effect on the ions, the $[Na^+]$ and $[K^+]$ levels were essentially the same as the perfused controls.

Significance to Bio-medical Research and the Program of the Institute: The finding that ouabain requires a pretreatment period to block NE uptake completely, explains discrepancies in reports from other laboratories. Ouabain appears to block the uptake of NE secondarily to a marked reduction of intracellular K^+ and to an increase in intracellular Na^+. Previous studies from this laboratory have shown an absolute Na^+ requirement for the uptake and storage of NE, and that this action of Na^+ is blocked by K^+. It was concluded that NE has a high affinity for a membrane carrier in the presence of Na^+ and a low affinity in the presence of K^+. It is possible, therefore, that ouabain blocks the uptake of NE indirectly by changing the intracellular levels of Na^+ and K^+ so that the carrier-NE complex does not readily dissociat The relatively poor ability of ouabain to release NE from its storage sites suggest that uptake and release are separate processes and that ouabain acts mainly on membrane pump.

Proposed Course of Project: The action of ouabain on the uptake of various amines will be investigated.

Honors and Awards: None

Publications: None

Serial No. NHI-135
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effect of Inorganic Ions on Storage and Release of H^3-NE from Sympathetic Neurons

Previous Serial Number: NHI-334

Principal Investigator: Dr. Donald F. Bogdanski

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.3
Professional:	0.3
Other:	0

Project Description:

Objectives: To study the role of various electrolytes in the process that takes up and stores neurohormones in nerve endings.

Methods Employed: Efflux of H^3-NE and uptake of H^3-NE was studied in rat heart slices using methods previously described.

Major Findings: Previous work has shown that Na^+ is an absolute requirement for the uptake and storage of norepinephrine (NE). Potassium ion has two effects. It facilitates the uptake and storage in low concentrations and antagonizes uptake and storage in high concentrations.

Our most recent experiments emphasized the uptake of H^3-NE. Uptake studies were run after preincubation of the heart slices in Krebs bicarbonate solution for 45 minutes, then transfer of the slices to fresh medium containing ascorbic acid. Under these conditions, slices did not take up H^3-NE in Na^+-free media made isotonic with sucrose. Uptake was gradually increased by increasing $[Na^+]$. Uptake was expressed as the ratio of tissue NE to bath NE. Plot of the reciprocal of this ratio against the reciprocal of $[Na^+]$ produced a straight line. With similar experiments, Kipnis and Parrish have indicated that this relationship indicates that 1 molecule of Na^+ is transported for each molecule of NE. Potassium ion in a concentration of 100 mM added to solutions containing various $[Na^+]$ inhibited uptake of norepinephrine. The reciprocals, plotted as before, crossed the y axis at the same point, indicating that potassium was a competitive inhibitor of Na^+.

We have also determined that uptake and metabolism of H^3-NE was decreased in Na^+-free media or in media containing ouabain. This finding suggests that the amine is taken up into the nerve endings of the heart before it can be metabolized.

Significance to Bio-medical Research and the Program of the Institute: These studies are attempts to understand fundamental processes in the uptake, storage and metabolism of biogenic amines in the sympathetic nervous system.

Proposed Course of Project: These studies will be continued according to areas of study revealed by previous research.

Honors and Awards: None

Publications: Bogdanski, D.F. and Brodie, B.B.: The Effects of Inorganic Ions on the Storage and Uptake of H^3-NE by Rat Heart Slices. J. Pharmacol. Exp. Ther., in press, 1968. Accepted with review.

Serial No. NHI- 136
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Isolated Nerve Ending

Previous Serial Number: NHI-353

Principal Investigator: Dr. Anja Tissari, Dr. Donald F. Bogdanski

Other Investigator: None

Cooperating Unit: Dr. Tissari is an International Fellow

Man Years:

Total:	1.2
Professional:	1.2
Other:	0.0

Project Description:

Objectives: To study the storage, uptake and synthesis of amines in isolated nerve endings (synaptosomes).

Methods Employed: Synaptosomes were isolated and re-suspended in various solutions of inorganic ions for study as previously described.

Major Findings: Preliminary findings reported last year were amplified and extended. The general ionic requirements for uptake and normal storage are the same in synaptosomes, as previously reported, for rat heart slices. That is, Na^+ is absolutely required for uptake and storage of norepinephrine (NE) and serotonin (5-HT). Norepinephrine, previously injected into the cerebral ventricles of rats, disappeared from synaptosomes (incubated in Krebs bicarbonate solution) at a rate corresponding to a half-life ($t\frac{1}{2}$) of 210 minutes. In the Na-free solution made isotonic with sucrose, $t\frac{1}{2}$ = 70 minutes. The change was progressive as Na^+ was subtracted. Storage of C^{14}-5-HT was also Na^+ dependent but the acceleration in the rate of efflux as Na^+ was removed was not as great.

The uptake of H^3-NE and C^{14}-5-HT was also Na^+ dependent. We used two criteria for uptake. 1) Accumulation of amine and 2) accumulation of amine plus total deaminated metabolite in the tissue and medium. Generally, with the synaptosome suspension of equal concentration (protein content) 5-HT was metabolized much more than NE.

372

Potassium, although not absolutely required, facilitated the action of Na^+ in uptake and storage. The efflux of amine was greater in K^+-free media (containing normal $[Na^+]$ than in normal Krebs solution, although the change was slight. Uptake was more dependent upon K^+, although none occurred in the absence of Na^+.

Using metabolites as a measure of uptake, we found that although the ouabain and reserpine blocked the accumulation of NE, ouabain markedly inhibited the formation of metabolites whereas while reserpine did not. This indicates that uptake of amine was blocked by ouabain, while reserpine permitted uptake and metabolism.

All the data parallel similar data accumulated in the study of the Na^+-dependent uptake of amino acids and sugars. We have adopted the hypothesis of Crane for the uptake of sugars in rabbit intestine as a model for amine uptake. Basically, this hypothesis requires the asymmetric distribution across the plasma membrane of Na^+, K^+ and a carrier having a high affinity for NE. The affinity is controlled by $[Na^+]$. Transport is not directly energy dependent through ATPase but is rather dependent upon ion gradients. An alternative hypothesis, expressed in the literature, is that energy of the ATPase system provides the energy for uptake. In order to differentiate the alternatives, we have determined that the uptake-blocking effect of ouabain (an ATPase inhibitor) is time dependent. Uptake is maximally blocked after preincubation for up to 15 minutes. Secondly, the uptake-blocking effect of Na^+-free media is reversible for 5 minutes. During this time, if ATPase is completely blocked by ouabain, the uptake follows its usual course upon the addition of Na^+. Thus, only the addition of Na^+ is required to restore uptake despite the prior blockade of ATPase.

Significance to Bio-medical Research and the Program of the Institute: This study will aid in the understanding of the molecular events involved in the uptake and storage of neurohormones by nerve endings. Such processes control many bodily functions, since these are controlled by nervous activity

Proposed Course of Project: These studies will continue along the lines set forth and as indicated by future findings.

Honors and Awards: None

Publications: Bogdanski, D.F., Tissari, A. and Brodie, B. B.: Role of sodium, potassium, ouabain and reserpine in uptake, storage and metabolism of biogenic amines in synaptosomes. Life Sci., 7: 419, 1968.

373

Serial No. NHI-137
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effect of Ouabain and Na^+ / K^+ on ATPase of Rabbit Synaptosomes

Previous Serial Number: None

Principal Investigators: Dr. Peter Schonhofer
Dr. Donald F. Bogdanski

Other Investigators: None

Cooperating Units: Peter Schonhofer is a NIH International Post-doctoral Fellow

Man Years:

Total:	0.3
Professional:	0.3
Other:	None

Project Description:

Objectives: According to the carrier model of Crane et al. uptake of sugars and amino acids is governed by the presence, uptake and extrusion of Na^+ in cells. Experiments in this laboartory have shown that this model for uptake into cells may also be applied to the uptake of norepinephrine and 5-hydroxytryptamine into synaptosomes. Uptake only occurs if there is a Na^+ gradient between outside and inside of the synaptosomes. The energy provided by ATPase is apparently not necessary for this process because uptake also occurs when the ATPase is blocked by ouabain.

Our experiments were designed to determine whether Na^+-K^+ATPase, which maintains the Na^+/K^+ gradient, was complete under the conditions of the uptake experiments. To determine the role of Na^+ and K^+ in the action of ouabain, the effect of these ions on the inhibition of the ATPase by this compound was also investigated.

Methods Employed: Rabbit brain stem synaptosomes were prepared by differential centrifugation according to Rodrigez de Lores Arnaiz et al. ATPase activities were measured by the release of inorganic P. The Na^+/K^+ dependent ATPase activity was determined by subtracting the activity obtained in the presence of the optimal Mg^{++} concentration alone from that obtained with Mg^{++}, Na^+ and K^+ present.

374

Major Findings: Ouabain completely blocks the Na^+-K^+ dependent ATPase activity in the synaptosome fraction at concentrations of 10^{-5} M. At higher concentrations ouabain inhibits total ATPase activity about 55-60%. Since the Na^+-K^+ dependent ATPase only accounts to 45% of the total ATPase activity, these findings indicate that ouabain also inhibits a part of the ATPase which is independent of Na^+-K^+.

The inhibition of the Na^+-K^+ dependent ATPase occurs very rapidly. With concentrations of 10^{-4} to 10^{-5} M ouabain no time lag was found if the compound was added at 0 time or if it was preincubated 5 min. For lower concentrations the results show slight differences in the values at 2 min as compared with those of 5 min preincubation, but later time points show no differences.

Ouabain is also effective in media which do not contain Na^+ and K^+, suggesting that the binding of the compound to the ATPase is not dependent on the presence of Na^+ or K^+. When Na^+ or K^+ or both ions plus substrate are added at 0 time (after 5 min preincubation with ouabain), no differences are found as compared to incubation with substrate plus all ions present at the time ouabain was added.

These findings give support to the work in this laboratory which showed that uptake of norepinephrine and 5-hydroxytryptamine is dependent on the flow of Na^+ into the cell. Regarding the carrier, there is no difference whether the Na^+ gradient is maintained by the extrusion of Na^+ out of the cell, affected by the Na^+-K^+ dependent ATPase, or by addition of Na^+ at 0 time. If the Na^+ pump is blocked by ouabain immediately, the addition of Na^+ to the mediums results in an uptake until equilibrium between inside and outside of the synaptosomes is reached.

Significance to Bio-medical Research and the Program of the Institute: The mechanism of uptake of transmitter substances into the nerve endings is not yet completely understood. For the discussed carrier model the energy requirement is not in the uptake mechanism, but in the extrusion of the Na^+. The findings with the ATPase and the effect of ouabain confirm this model.

Proposed Course of Project: No further studies are anticipated.

Honors and Awards: None

Publications: None

Serial No. NHI- 138
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effect of Chronic Lithium Treatment on Storage of Brain Norepinephrine

Previous Serial Number: None

Principal Investigators: Dr. Donald F. Bogdanski
Dr. Kenneth Greenspan

Other Investigators: None

Cooperating Units: Dr. Greenspan is in the Laboratory of Clinical Science, NIMH

Man Years:

Total:	0.1
Professional:	0.1
Other:	0

Project Description:

Objectives: To determine the effect of chronic lithium treatment on the storage of brain norepinephrine.

Methods Employed: Rats were given Li^+ injections in doses of 0.1 to 0.3 nmole/kg i.p. for a total of 10 days. On the last day, H^3-NE was injected into the brain ventricle, the rats were killed at various time intervals thereafter and assayed for radioactive H^3-NE and metabolite.

Major Findings: Results are preliminary at this time. In control animals H^3-NE disappeared at a rate corresponding to a half-life ($t_{\frac{1}{2}}$) of 6 hours. Similar results were obtained in animals given 0.1 nmole/kg Li^+. The $t_{\frac{1}{2}}$ of H^3-NE was about 5 hours and 2 hours in animals given 0.2 and 0.3 nmoles/kg Li^+. In the last group, this storage deficit was accompanied by an increase in the brain levels of deaminated, O-methylated metabolite, indicating that the amine was taken up in the brain but rapidly metabolized by monoamine oxidase. Presumably, Li^+ produces a deficiency in the storage process, resulting in a more rapid destruction and turnover of the amine.

Significance to Bio-medical Research and the Program of the Institute: These studies may aid in the understanding of the possible role of brain amines in the cause and treatment of the manic-depressive syndrome. These studies also may shed light on the role of inorganic electrolytes on the

376

storage of amines in nerve endings.

Proposed Course of Project: The studies will continue as indicated to complete the primary objectives.

Honors and Awards: None

Publications: None

Serial No. NHI- 139
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Central Effects of Norepinephrine and Serotonin

Previous Serial Number: NHI-347, 348 and 349 (now combined)

Principal Investigators: Dr. Zbigniew Herman
Dr. Donald F. Bogdanski

Other Investigators: None

Cooperating Unit: Dr. Herman was supported by a Riker Fellowship

Man Years:

Total:	0.5
Professional:	0.5
Other:	0.0

Project Description:

Objectives: To investigate and determine the functional role of serotonin (5-HT) and norepinephrine (NE) in the brain.

Methods Employed: Drugs were injected into the brain ventricles. Norepinephrine and metabolites were estimated by methods previously described from this laboratory.

Major Findings: BW 392C60 was used as a tool to study the physiological role of NE in rat brain. BW 392C60 is known to be a peripherally acting sympatholytic drug. It acts by preventing the release of NE from nerve ending during normal physiological activity. We have injected H^3-NE into brain ventricles and studied the disappearance of the amine and its metabolites alone and under the influence of drug combinations. Reserpine increases the rate of disappearance of NE and the rate of metabolism of H^3-NE. BW 392C60 slows the disappearance and the rate of metabolism of H^3-NE when given alone or before reserpine. Pargyline, a monoamine oxidase inhibitor (MAOI), does not prevent the release of NE by reserpine. However, most of the radioactivity is recovered as normetanephrine, indicating that the amine has been released and O-methylated.

In accord with the view that BW 392C60 prevents the release of NE, the drug does not cause excitement when given before reserpine, although it is as good a MAOI as pargyline. In contrast, BW 392C60 does not prevent the release of serotonin (5-HT) or dopamine (DA) by reserpine.

378

By preventing the release of NE in the brain, BW may be used to study the central actions of NE by causing a functional deficit of NE in the brain. The pharmacological actions of BW 392C60 are opposite to the normal actions of NE. BW 392C60 causes sedation, potentiates hexobarbital anesthesia and produces hypothermia. These effects indicate that normally NE maintains alert behavior and prevents body temperature from falling too low.

Continuing experiments begun last year, we found that intraventricular injection of 5-HT is sedative in animals in which NE has been depleted by α-methyl-tyrosine. Conversely, NE is excitatory in animals given p-Cl phenylalanine in order to deplete the brain to 5-HT. These experiments indicate that 5-HT and NE have opposite actions centrally.

Significance to Bio-medical Research and the Program of the Institute: These studies may aid in the understanding of the role of 5-HT and NE in the brain function.

Proposed Course of Project: Work has temporarily been discontinued due to the departure of Dr. Herman. Additional experiments designed to study the role of brain amines will be resumed shortly.

Honors and Awards: None

Publications: None

319

Serial No. NHI-140
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of Action of Parachlorophenylalanine

Previous Serial Number: None

Principal Investigators: Dr. Henry Bourne
Dr. James Davis

Other Investigators: None

Cooperating Units: Drs. Bourne and Davis are Research Associates in the Pharmacology-Toxicology Program, NIGMS

Man Years:

Total:	0.2
Professional:	0.2
Other:	0.0

Project Description:

Objectives: Parachlorophenylalanine (PCP) has been shown in this and other laboratories to inhibit the synthesis of 5-HT by blocking the activity of the rate-limiting biosynthetic enzyme, tryptophan hydroxylase. Although this blockade, *in vivo* and *in vitro*, is virtually complete (greater than 90% in rat brain), brain levels of 5-HT fall comparatively slowly after the drug is given, with a T 1/2 of approximately 16 hours. This fact seems to be in contradiction to the measurements of turnover rate of 5-HT in brain, in this and other laboratories, which indicate a much shorter T 1/2 of the order of less than one hour. If the only action of PCP were a complete block of synthesis, brain levels of 5-HT should fall much faster than they do. These experiments examined several hypothetic additional actions of PCP.

Methods Employed: 5-HT was measured by the method of Bogdanski *et al*.

Major Findings: (1) One possibility is that PCP blocks the known pathway for 5-HT degradation, monoamine oxidase. If this is so, a monoamine oxidase inhibitor ought not to appreciably affect the depletion of 5-HT by PCP. We found, instead, that a large dose of the monoamine oxidase inhibitor pargyline, given four hours after PCP, was able to block almost completely the further depletion of 5-HT.

(2) Another possibility is that the 5-HT fluorescence extracted from brain after PCP is not actually 5-HT at all, but some other substance,

either a 5-OH-indole metabolite of 5-HT, or a metabolite of the drug, PCP. This possibility was examined in two ways. First, counter-current distribution of the 5-HT fluorescence after PCP indicated no difference from fluorescence in controls. Second, a possibly more specific method for measurement of 5-HT, the ninhydrin method of Snyder and Axelrod, was used to examine 5-HT levels after PCP. Although the ninhydrin method gave exactly the same levels as the Bogdanski method in control brains, after PCP the fluorescence by the ninhydrin method was termendously increased. This fluorescence had slightly different activation and emission spectra from the 5-HT-ninhydrin product, and was associated with a slight yellow color forming in the reaction mixture.

(3) A brief attempt at determining the nature of this fluorescent substance, which could be a metabolite of tryptophan, 5-HT, or PCP, was unsuccessful. The fluorescence was not due to: a) PCP itself, which did not form a fluorescent product with ninhydrin; b) parachlorophenylethylamine, the decarboxylated product of PCP, which reacted with ninhydrin to give fluorescent spectra similar to the unknown substance, but which exhibited completely different oil-water partition characteristics at high pH; 3) nor, for similar reasons, 6-chloro-indole, 5-methoxy-indoleamine, or any aromatic acids. It was concluded that the substance, whatever it may be, is quite similar to 5-HT in its lipid solubility, pH partition curves and reaction with ninhydrin.

Significance to Bio-medical Research and the Program of the Institute: Discovery of a fluorescent product in rat brain after PCP may further elucidate the mechanism of action of this important drug.

Proposed Course of Project: Further attempts to characterize the fluorescent product in brain extracts after PCP treatment are planned.

Honors and Awards: None

Publications: None

381

Serial No. NHI-141
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Synthesis of Brain Serotonin from Radioactive Tryptophan

Previous Serial Number: None

Principal Investigators: Dr. Henry Bourne
Dr. James Davis

Other Investigators: None

Cooperating Units: Drs. Bourne and Davis are Research Associates in the Pharmacology-Toxicology Program, NIGMS

Man Years:

Total:	0.6
Professional:	0.6
Other:	0.0

Project Description:

Objectives: The ability of brain tissue to synthesize serotonin from tryptophan in vivo has been established, with isolation of the two enzymes involved--tryptophan hydroxylase and 5-OH-tryptophan decarboxylase. The actual rate of synthesis in vivo has been measured in this laboratory by an indirect method, involving the use of monoamineoxidase inhibitors. These experiments attempted to measure in vivo synthesis of the brain amine without use of a drug.

Methods Employed: Serotonin was measured by the fluorescent method of Bogdanski et al. Tryptophan was estimated by the fluorescent method of Udenfriend. Radioactive serotonin was separated from tryptophan and 5-HTP by use of an IRC-50 column.

Major Findings: (1) After injection of a tracer dose of H^3-tryptophan intravenously, tryptophan specific activity in plasma dropped rapidly, with an initial T 1/2 of approximately 10 minutes. After about 30 minutes, however, the plasma specific activity began to drop more slowly, with a T 1/2 of about 120 minutes. Tryptophan specific activity in brain, however, declined more slowly: the brain specific activity is initially less than that in plasma, but at two hours is more than double the plasma values.

(2) Serotonin specific activity in brain rose rapidly and reached a peak at 30 minutes after injection of H^3-TP. Specific activity of serotonin never

reached more than one half that of the brain tryptophan, although from one hour on plasma TP and Brain 5-HT specific activities were equal.

(3) Parachlorophenylalanine is a drug thought to inhibit synthesis of 5-HT by inhibition of the rate-limiting biosynthetic enzyme, tryptophan hydroxylase. After pretreatment with this drug there was virtually no detectable synthesis of labelled 5-HT from labelled TP in the rat brain, although brain and plasma specific activities of TP remained unchanged. This experiment confirms the idea, heretofore attacked only by indirect means, that parachlorophenylalanine does indeed cause a decrease in brain 5-HT levels by blocking synthesis of the amine.

Significance to Bio-medical Research and the Program of the Institute: The function of brain serotonin, although not yet clear, can be further elucidated by measurement of its rate of synthesis *in vivo*. In addition, the theory proposed for the mechanism of action of parachlorophenylalanine--a new drug used clinically for treatment of the carcinoid syndrome--has been confirmed.

Proposed Course of Project: We plan to use this method of measuring synthesis rates of serotonin to physiological conditions, such as sleep and heat stress, in which serotonin metabolism should be affected.

Honors and Awards: None

Publications: None

Serial No. NHI- 142
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Uptake of Metaraminol and Release of Norepinephrine

Previous Serial Number: None

Principal Investigators: Dr. James Davis
Dr. Henry Bourne

Other Investigators: None

Cooperating Units: Drs. Davis and Bourne are Research Associates in the Pharmacology-Toxicology Program, NIGMS

Man Years:

Total:	0.8
Professional:	0.8
Other:	0.0

Project Description:

Objectives: Earlier studies in this laboratory had established the time-course of heart levels of metaraminol after intravenous injection into rats, and the consequent release of norepinephrine. To elucidate further the mechanism of the norepinephrine release from sympathetic neurons we examined the effects of reserpine and desipramine on metaraminol uptake and norepinephrine levels in vivo.

Methods Employed: Endogenous NE was measured by routine methods (Chang et al.). MA was estimated by the fluorimetric method of Shore and Alpers.

Major Findings: 1) After metaraminol injection into rats at doses of 50 to 1000 micrograms per kg, heart norepinephrine levels drop rapidly for three to five hours. The larger doses, e.g. 500 micrograms per kg, achieve 90% depletion of NE, while smaller doses achieve correspondingly less depletion. When the depletion is completed, however, NE levels remain constant for 15-20 hours, and then begin to return slowly to normal. During both the depletion phase and the low plateau, however, metaraminol levels in the same tissue remain absolutely constant, and characteristic for any given dose of the drug.

2) Pretreatment of rats with reserpine (7 mg/kg, 5 hours) and desipramine (10 mg/kg, 1 hour) before metaraminol injection changed the levels of drug in the heart dramatically. Although initial levels of MA (five minutes) in

the pretreated animals are not much less than in controls, there is a swift disappearance of drug, so that after one hour virtually none of the MA remains. According to current views of the mechanism of action of desipramine and reserpine on peripheral sympathetic neurons, the combination of the two drugs at these dose levels should completely block both "uptake" and storage of amines by sympathetic neurons in the heart, by inhibiting both the membrane pump and the granule storage mechanism. If this is true, then levels of MA in the presence of the two drugs may represent a non-specific, perhaps non-neuronal uptake of MA, which presumably was present also in control animals, although masked by the specific storage of the drug by intact neurons. From these results it may be inferred that the "specific" uptake by neurons is a process that is completed in normal animals one hour after injection of MA.

It is noteworthy that although neuronal uptake is completed in one hour, NE depletion is not--instead, it continues for another four hours. These results contradict the view that MA acts simply by stoichiometric displacement of the endogenous neurotransmitter. At least at early times after drug injection, both the injected drug and the endogenous amine are present in the heart.

Significance to Bio-Medical Research and the Program of the Institute: Studies of the mechanism of action of MA, a clinically important vasopressor drug, will increase or improve knowledge of its clinical action, and provide more information about storage and release of norepinephrine.

Proposed Course of Project: Further studies on uptake and storage of MA and other amines are under way in this laboratory.

Honors and Awards: None

Publications: None

385

Serial No.- NHI-143
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of reserpine on the glycoprotein of the Submaxillary gland

Previous Serial Number: NHI-320

Principal Investigators: Dr. Palmer W. Taylor
Dr. Elwood O. Titus

Other Investigator: Mrs. Patricia R. O'Keeffe

Cooperating Unit: Dr. Taylor is a Research Associate in Pharmacology-Toxicology Training Program, NIGMS

Man Years:

Total:	1.5
Professional:	0.9
Other:	0.6

Project Description:

Objectives: Previous findings concerning reserpine-induced alterations in the structure of proteins from the submaxillary gland suggested that alterations in glycoprotein structure occur as a direct action of the drug and are not secondarily mediated through the usual central and autonomic actions of reserpine. Since glycoproteins are important components of cellular membranes and appear to be localized in synaptic areas, induced changes in their structure could be responsible for the long-lasting response that is characteristic of reserpine.

Methods Employed: Standard methods of protein fractionation and carbohydrate assay were employed.

Major Findings: A previous report has shown that one day after the administration of reserpine to rats a large fraction of the proteins (including the neuraminic acid containing glycoproteins) of the submaxillary gland becomes soluble in trichloracetic acid, which is under normal circumstances a protein precipitant.

In the current year efforts have been made to identify the changes in submaxillary protein that are induced by reserpine. For the most part these have been carried out with the supernatant fraction obtained by high speed centrifugation of aqueous extracts from the submaxillary glands of normal and reserpine pretreated rats.

A. Gel Filtration Studies. Chromatography on Sephadex G-200 at pH 7.0 disclosed no differences between preparations from normal and pretreated

386

animals. In both cases all of the neuraminic acid emerged in the excluded fraction which contained the proteins of high molecular weight.

If similar chromatography was performed with Sephadex at pH 2.0 using the acid-soluble material obtained from the glands of reserpinized rats, all of the protein occurred in a single peak of high molecular weight. (The amount of high molecular weight protein extracted by acid from control preparations was insignificant.) Removal of acid by dialysis at neutral pH and subsequent electrophoresis showed the fraction of apparent high molecular weight to be in fact an aggregate of at least three proteins of variable molecular weight.

B. Titration Experiments. Titration of carefully dialyzed supernatants showed that preparations from reserpinized animals exhibited an enhanced adsorption of H^+. This increment was 6 times greater than the total neuraminic acid equivalents in the preparation and occurred in the pH range 3.75 to 4.25, which is too high to represent the titration of neuraminic carboxyls. The enhanced adsorption of H^+ would be consistent with a diminished association between macromolecules according to the Linderstrom-Lang theory of protein association. It may also be that the proteins modified by reserpine contain enhanced amounts of glutamyl or aspartyl residues.

C. Light Scattering Experiments. Changes in the intensity of scattered light offer a sensitive indicator of incipient precipitation or aggregation as acid is added to solutions of protein mixtures. Reserpine-induced changes in the structure of a single protein might also be expected to reveal themselves as changes in the rate at which acidification produced aggregation or precipitation. Preparations from control and reserpine-treated rats showed striking differences when the intensity of scattered light was plotted with respect to either added calcium or hydrogen ions. The effects of the added calcium ion were independent of pH changes brought about by the addition of the calcium salts.

Fractionation of control and reserpine pretreated preparations has shown that most of the differences in light scattering can be assigned to a single protein which can be separated from other components of the submaxillary gland by chromatography on Sephadex and DEAE cellulose. These procedures reveal no differences in chromatographic properties of the fractions isolated from control or treated animals. The protein thus isolated from reserpine-treated animals, however, shows some difference in behavior on disc gel electrophoresis and very dramatic reduction in its capacity to aggregate. The isolated protein has no neuraminic acid.

Significance to Biomedical Research and the Program of the Institute: Reserpine, which has found wide use both clinically and in pharmacological research, is unique among agents affecting the autonomic nervous system because of its long duration of action. The molecular basis for this is still unknown. Since changes in protein synthesis or structure may be involved, these studies on induced changes in glycoprotein association and structure may be useful in elucidating the mechanism of action of this drug.

Proposed Course of Project: Additional characterization of the purified reserpine sensitive protein will be continued to obtain information on the chemical and physical structure. Attempts will be made to prepare fluorescent antibody to the isolated protein. The product will be used to determine by immunochemical means whether the reserpine sensitive protein of submaxillary gland can be detected in reserpine sensitive structures such as sympathetic nerve endings.

Honors and Awards: None.

Publications: None.

Serial No.- NHI-144
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Relationship between submaxillary gland function and autonomic innervation

Previous Serial Number: None.

Principal Investigators: Dr. Palmer W. Taylor
Dr. Sidney Wolfe

Cooperating Unit: Dr. Wolfe is a Clinical Associate of the Metabolic Diseases Branch, NIAMD

Man Years:

Total:	0.3
Professional:	0.3
Other:	None

Project Description:

Objectives: As an outgrowth of our investigations with reserpine it became apparent that the submaxillary gland provided an ideal organ to examine the relationship between autonomic innervation and secretory function. Unilateral sympathetic denervation or section of the chorda tympani permits the observation of a normally innervated control gland in each animal. The surgical procedures also circumvent certain problems due to multiple sites of action and incomplete effects that are encountered with a pharmacologic autonomic block. Conventional surgical procedures were used for parasympathetic denervation and parasympathetic decentralization by section of the chorda tympani. Conventional colorimetric methods for the determination of the carbohydrate components of glycoproteins were used.

Major Findings: 1) Calcium and Magnesium Content and Turnover with Denervation: Four days after sympathetic denervation the glandular Ca and Mg respectively averaged 152% and 146% of their contralateral control gland level. A steady state turnover rate of 4.5 μEq/g of tissue is obtained from tracer studies. This value is 65% of the contralateral control gland.

2) Changes in Glandular Ca, Mg and Glycoprotein Caused by a Secretory Stimulus: Since saliva spreading is an important means of thermoregulation in the rat, a thermal stress should evoke a central stimulus for salivation. Rats placed in a metabolic chamber at 105°F and 55% relative humidity show a rapid loss of glandular Ca such that after one hour their levels are 55% of control. Only a small additional decrease is noted over the next 5 hours.

This response seems to be specific for Ca since no depletion of glandular Mg is found. Also no changes in neuraminic acid or hexosamine which serve as markers for glycoprotein are evident over a six-hour period. The sympathetically denervated gland responded surprisingly well to the stimulus as a slightly greater percentage depletion of Ca results in this gland at each point in the time course.

Tracer studies show an increased influx of Ca into the gland under these conditions. This indicates that glandular activity can stimulate the transfer of cations into the gland.

Proposed Course of Project: Similar studies on glandular Ca levels and turnover both in the normal steady state and under thermal stress are planned for rats which have had their chorda tympani sectioned. Completion of these studies should further delineate the relative role of parasympathetic and sympathetic function in secretion. A mathematical model has been developed which permits the calculation of the kinetic constants for calcium influx and efflux from data on levels and turnover rates in the submaxillary gland. Determinations of these fluxes will be carried out both in the normal steady state and after experimental perturbation of the calcium levels.

Significance to Biomedical Research and the Program of the Institute: Patients with cystic fibrosis have an increased Ca content and abnormal glycoprotein levels in their secretions. Knowledge concerning the turnover of the above glandular components under various conditions may be helpful in determining if the basic defect is related to autonomic disfunction.

Honors and Awards: None.

Publications: None.

Serial No. - 145

1.Chemical Pharmacology
2.Organic Chemistry
3.Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effect of reserpine on the divalent cation content in the submaxillary gland

Previous Serial Number: None

Principal Investigators: Dr. Palmer W. Taylor
Dr. Sidney Wolfe

Cooperating Unit: Dr. Wolfe is a Clinical Associate in NIAMD

Man Years: 0.6

Project Description:

Objectives: An unusually high Ca^{++} content (20 μEq/gram of tissue) is found in the submaxillary gland of rats. Although high concentrations of this ion are secreted into the saliva, little is known about the mechanism of its transport into the gland or the state in which it is stored or bound. The changes induced in glandular glycoproteins by reserpine offer an interesting opportunity to examine the relationship between glycoproteins and Ca^{++} storage.

Methods Employed: Standard ultrafiltration and radioisotope tracer techniques were used. Ca^{++} and Mg^{++} were assayed by atomic absorption.

Major Findings: After a single dose of reserpine the glandular Ca content increased from 22 ± 2 to 56 ± 3 μEq/gram of tissue. Mg also increased from 28 ± 2 to 48 ± 2 μEq/gram of tissue. A reduction in K^{+} levels amounting to 10 μEq/gram of tissue did not compensate for the changes in Ca and Mg. The time course of this response, which closely paralleled the changes in glycoprotein, exhibited a slow onset, became maximal in 30 hrs and disappeared in 70 hrs. Significant changes could be obtained with single doses as low as 0.03 mg/kg. Essentially all of this increase was confined to the 100,000 x g supernatant when glands were homogenized in low ionic strength buffer and the homogenates centrifuged. Here Ca rose from 8 to 38 μEq/gram of tissue and Mg from 12 to 36 μEq/gram of tissue. It appears that these cations are weakly associated with acidic proteins since ultrafiltration experiments show a preferential binding of these divalent cations. This binding, which is substantially greater in glands from reserpine pretreated animals, can be greatly reduced if ultrafiltration is conducted at high ionic strength.

Tracer studies employing Ca^{47} reveal that this increased calcium level is a consequence of an inhibited efflux from the gland. A steady state turnover rate of 5.8 μmoles/gram of tissue/hr is found for control glands. This is in close agreement with an influx rate of 6.1 μmoles/gram of tissue/hr calculated from the rate of labeling of the gland and the mean of the precurso

3/11

(serum) specific activity at short time intervals after injection. After the administration of reserpine no increase in the influx rate into the gland is observed. However, the calculated efflux rate between 5 and 30 hrs after reserpine administration is reduced to 1.4-2.1 μmoles/gram of tissue/hr.

Sympathetic denervation causes a smaller but significant increase in glandular calcium and magnesium. However, reserpine can elicit a further rise in Ca in the previously denervated gland. This suggests a direct effect of this drug on the gland as was observed with the glandular glycoprotein alterations.

This effect on divalent cations has thus far been specific for the submaxillary gland as no change in tissue Ca or Mg was detected in the lacrimal gland, muscle, liver or serum after reserpine administration.

Significance to Biomedical Research and the Program of the Institute: Ca and Mg have an important role in secretion and autonomic function. Drugs which alter their tissue levels will be important aids in investigating the mechanism by which these ions are transported and secreted.

Proposed Course of Project: Further studies on the relationship between Ca and glycoprotein structure are planned.

Honors and Awards: None.

Publications: None.

Serial No.- NHI-146
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Enzymatic Mechanism of Membrane Transport

Previous Serial Number: NHI-324

Principal Investigators: Dr. Colin F. Chignell
Dr. Elwood O. Titus

Other Investigators: Mrs. Donnas K. Starkweather
Mrs. Loekie Van de Ven

Cooperatings Units: Dr. Chignell is a Research Associate in the Pharmacology Program of NIGMS. Mrs. van de Ven is a guest worker supported by the National Cystic Fibrosis Foundation.

Man Years:

Total:	1.1
Professional:	0.4
Other:	0.7

Project Description:

Objectives: Part I. Microsomal fractions from brain, kidney and other tissues contain a magnesium-dependent adenosine triphosphatase (ATPase) which is stimulated by the simultaneous presence of sodium and potassium ions. There is evidence that this enzyme is identical with, or an integral part of, the active cation transport mechanism located in cell membranes.

Studies in our own and other laboratories have shown that the terminal phosphate of γ-labelled ATP^{32} may be transferred to microsomal $(Na^+ + K^+)$-ATPase preparations in a Na^+-dependent step. Since the subsequent addition of K^+ to the medium results in a rapid loss of inorganic phosphate, a two-step mechanism for the hydrolysis of ATP by $(Na^+ + K^+)$-ATPase has been postulated:

$$(1)\quad ATP + E \underset{}{\overset{Na^+Mg^{++}}{\rightleftharpoons}} E \sim P + ADP$$

$$(2)\quad E \sim P + H_2O \xrightarrow[k_1]{K^+} E + P_i$$

Labelled phosphoprotein prepared by TCA precipitation from reaction mixtures containing Na^+, but not K^+, has been found to lose inorganic phosphate

393

at high pH and in the presence of hydroxylamine. Since these are reactions characteristic of acyl phosphates, it has been suggested that phosphorylation of an acyl group of the protein plays an intermediary role.

To determine the precise role of an enzyme bound acyl phosphate in the hydrolysis of ATP by microsomal ($Na^+ + K^+$)-ATPase we have investigated the interaction of this enzyme with hydroxylamine and its derivatives.

Part II. Recent radiation inactivation techniques indicate that the molecular weight of guinea pig microsomal ($Na^+ + K^+$)-ATPase is about 190,000. Using this figure it appears that microsomal preparations contain from 5-20% of the pure ($Na^+ + K^+$)-ATPase depending on their enzymatic activity. Attempts to purify the enzyme by classical biochemical techniques have resulted in a reduction or total loss of ($Na^+ + K^+$)-ATPase activity. Part of the problem may be that ($Na^+ + K^+$)-ATPase is a multicomponent system which is dissociated into inactive subunits by purification procedures. In an attempt to identify the protein or proteins which comprise ($Na^+ K^+$)-ATPase we have fractionated microsomes from beef and rat brain and rat kidney using disc gel electrophoresis.

Methods Employed: Part I. Beef brain microsomes were prepared by differential centrifugation of desoxycholate treated homogenates (Eur. J. Biochem. 1: 334, 1907). ATPase activities were measured in the presence of optimal concentrations of Na^+, K^+, Mg^{++} and ATP. The ($Na^+ + K^+$)-ATPase activities were calculated by subtracting the activity obtained in the presence of Mg^{++} alone from that obtained with Mg^{++}, Na^+ and K^+.

The kinase activity (reaction 1) was measured by incubating the microsomes at 37°C with Na^+, Mg^{++} and ATP^{32}. The phosphoprotein was precipitated with TCA and isolated by filtration on Millipore filters. The readioactivity was determined by standard scintillation counting techniques.

Part II. Microsomes were dissolved in a phenol:acetic acid:urea mixture (2:1:1) and fractionated over disc electrophoresis columns by the method of Cotman and Mahler. When labelled microsomes were used the gel was sectioned and the slices dissolved in hydrogen peroxide, then counted in a liquid scintillation counter.

Major Findings: Part I. Hydroxylamine (NH_2OH) and its N-methyl analog (CH_3NHOH) inhibited the ($Na^+ + K^+$)-ATPase activity of desoxycholate treated microsomes (cf. results with sodium iodide treated microsomes). Inhibition was not a simple function of concentration (Table 1). At 10^{-2} M both NH_2OH and CH_3NHOH produced about 50% inhibition of enzyme activity which was not then further affected by a ten-fold increase in inhibitor concentration. Increasing NH_2OH concentration above 10^{-1} M caused a further decrease in enzyme activity whereas a similar increase in CH_3NHOH concentration resulted in a partial reversal of inhibition. Kinetic studies showed that when enzyme activity was examined as a function of K^+ concentration, hydroxylamine inhibition was non-competitive at concentrations of 2×10^{-3} M and below, becoming mixed at 10^{-2} M and above.

394

Table 1

Inhibitor concentration (M)	$(Na^+ + K^+)$-ATPase Activity (% of control)	
	NH_2OH	CH_3NHOH
10^{-5}	98.2	92.7
10^{-4}	90.3	80.6
10^{-3}	74.9	61.0
10^{-2}	44.7	51.6
10^{-1}	45.7	50.7
8×10^{-1}	10.4	59.7

The addition of NH_2OH (0.8 M) to microsomes labelled in the presence of Mg^{++}, Na^+ and ATP^{32} resulted in a reduction in the level of labelling such that Mg^{++} control levels were reached in 30 seconds (Table 2). During this time the rate of inorganic phosphate production increased, suggesting that NH_2OH is not reacting chemically with the intermediate but is promoting the enzymatic breakdown of the intermediate in the same way as K^+ (see Objectives). This effect is probably due to contamination of the NH_2OH by NH_4^+, a cation which acts like K^+.

Table 2

	Addition (made at 10 sec)	P^{32} Incorporation at 40 sec (μμmoles/0.2 mg protein)	Pi released during 10-40 sec (mμmoles/ 0.2 mg protein)
Mg^{++} ATP^{32}	--	14.5	2.5
Mg^{++} Na^+ ATP^{32}	--	50.5	6.2
"	0.8 M NH_2OH	12.0	8.8
"	0.8 M CH_3NHOH	42.5	2.2

CH_3NHOH produced only a 25% decrease in the level of labelling in 30 seconds, while at the same time markedly reducing the rate of inorganic phosphate production (Table 2). However, microsomes which had been incubated with CH_3NHOH, Na^+, Mg^{++} and ATP still incorporated label when re-incubated with ATP^{32}. These results suggested that either CH_3NHOH was not reacting with the acyl phosphate or that reaction did occur but the acyl phosphate was not the intermediate in the hydrolysis of ATP by $(Na^+ + K^+)$-ATPase. Using CH_3NHOH it is not possible to differentiate between these alternatives.

Part II. Microsomes were separated into about 12-15 components by the disc gel electrophoresis technique. Two approaches were used to identify bands associated with $(Na^+ + K^+)$-ATPase activity. In the first microsomes were labelled with P^{32} by incubation with Mg^{++}, Na^+ and ATP^{32}. After fractionation the radioactivity was found to be associated almost exclusively with a well-

defined slow moving band. In a second approach the gel pattern of kidney microsomes from prenatal rats was compared with that from postnatal animals in which the $(Na^+ + K^+)$-ATPase activity was much higher. A slow moving band, identical with that which became labelled in the previous experiments, was found to increase in density in the postnatal rat kidney microsomes. Similar results were obtained with brains from 4- and 28-day old rats in which a similar increase in $(Na^+ + K^+)$-ATPase is observed. This technique then has enabled the identification of at least one protein band with $(Na^+ + K^+)$-ATPase.

Significance to Biomedical Research and the Program of the Institute: The transport of cations maintains the membrane potentials necessary for the function of nervous tissue, governs the access to the heart of actions which influence the strength of contraction and is important in kidney function. Although many drugs with cardiovascular effects act on membrane transport, the molecular basis for their action is not understood.

Proposed Course of Project: The interaction of various N-substituted derivatives of hydroxylamine with $(Na^+ + K^+)$-ATPase will be studied. At present $(Na^+ + K^+)$-ATPase is considered to hydrolyze ATP in two stages:

$$ATP + E \xrightarrow{Na^+ Mg^{++}} E \sim P + ADP$$

$$E \sim P + H_2O \xrightarrow{K^+} E + P_i$$

The first half reaction is probably associated with the slow moving protein identified above. To determine whether the same protein catalyzes the second reaction a different approach will be used. In the presence of potassium ions the Mg^{++}-dependent inactivation of microsomal $(Na^+ + K^+)$-ATPase is greatly increased. It is presumed that di-isopropyl phosphofluoridate (DFP) blocks the active site on the protein which is involved in the second reaction. Microsomes labelled with DFP^{32} in the presence of K^+ will therefore be fractionated and the localization of the radioactive label determined.

Honors and Awards: None.

Publications:

Chignell, C. F.: Effect of phenolphthalein and other purgative drugs on rat intestinal $(Na^+ + K^+)$-adenosine triphosphatase. Biochem. Pharmacol., in press.

Chignell, C. F. and Titus, E. O.: Effect of hydroxylamine and N-methylhydroxylamine on a $(Na^+ + K^+)$-adenosine triphosphatase from beef brain. Biochim. Biophys. Acta, in press.

396

Serial No. - NHI-147
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Use of antibiotics to study mechanisms of ion transport

Previous Serial Number: NHI-324

Principal Investigators: Dr. Charles E. Inturrisi
Dr. Elwood O. Titus

Cooperating Unit: Dr. Inturrisi is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Man Years:

Total:	0.75
Professional:	0.75
Other:	None

Project Description:

Objectives: The $(Na^+ + K^+)$-ATPase is an enzyme generally considered to be a major component of the cellular mechanisms of sodium and potassium transport. Hydrolysis of ATP by this enzyme occurs in two steps, the first of which is a Na^+-dependent phosphorylation of enzyme protein. The second is a K^+-requiring dephosphorylation. It is possible, although still uncertain, that the two steps are mediated by separate proteins functioning in association.

The $(Na^+ + K^+)$-ATPase can be prepared from many tissues, including bovine brain, as a microsomal fraction. This particulate fraction contains, in addition to the $(Na^+ + K^+)$-ATPase, a K^+-activated phosphatase which hydrolyzes acetyl phosphate or p-nitrophenyl phosphate. It has not been satisfactorily determined whether the K^+-phosphatase is a separate enzyme or actually represents the K^+-requiring portion of the $(Na^+ + K^+)$-ATPase. Preliminary results by Israel and Titus (Biochim. Biophys. Acta 139: 450, 1967) suggested that the antibiotic oligomycin would inhibit the $(Na^+ + K^+)$-ATPase but not the K^+-phosphatase. Oligomycin is not an inhibitor of the initial phosphorylation of protein. The purposes of the present project were: 1) to extend the observations with oligomycin and to determine if closely related macrolide antibiotics also are capable of inhibition of $(Na^+ + K^+)$-ATPase, 2) to study the kinetics of oligomycin inhibition in the hope that they would give some insight into the mechanism of action of the enzyme.

Methods Employed: The enzyme was prepared as described by Schoner et al.

397

(European J. Biochem. 1: 334, 1967). Incubation tubes contained the appropriate substrate - ATP^{32}, acetylphosphate, or p-nitrophenyl phosphate plus $MgCl_2$ to give a Mg/substrate ratio of 2.5, and 100 mM Tris-HCl, pH 7.4, in a final volume of 1.0 ml. The reaction was initiated by addition of 60-100 µg of enzyme. The $(Na^+ + K^+)$-ATPase activity was measured by the liberation of ^{32}Pi from ATP^{32} in the presence of 100 mM NaCl and 30 mM KCl.

The K^+-phosphatase activity was estimated by the increase in the rate of hydrolysis of acetylphosphate or p-nitrophenyl phosphate in the presence of 30 mM KCl.

Major Findings: The antibiotics oligomycin and rutamycin inhibit the $(Na^+ + K^+)$-ATPase but not the K^+-phosphatase in concentrations from 10^{-7} to 10^{-4} M. Structurally related antibiotics, including erythromycin, methymycin, amphotericin B and fungichromin did not inhibit the $(Na^+ + K^+)$-ATPase or the K^+-phosphatase.

Kinetic studies revealed that inhibition by oligomycin is "uncompetitive," a type of inhibition which ensues when the antagonist reacts with the enzyme substrate complex rather than with the enzyme alone. The enzyme activation by Na^+ is also uncompetitively inhibited by oligomycin, while the K^+ activation is noncompetitively inhibited, i.e., oligomycin altered the maximal rate, but not the affinity for K^+.

Significance to Biomedical Research and the Program of the Institute: Oligomycin is an inhibitor of the $(Na^+ + K^+)$-ATPase which appears to be much more specific than the cardiac glycosides. The latter are inhibitors of the K^+-phosphatases as well as ATPase. Thus oligomycin may prove to be an important tool with which to study the enzymatic basis of cation transport.

Proposed Course of Project: It will be of interest to determine what role Na^+ plays in conferring oligomycin sensitivity on the ATPase. Several studies have reported that Na^+ increases the binding of ouabain to the ATPase. Perhaps this is also true for oligomycin. This could explain why the K^+-phosphatase is not inhibited by oligomycin. Experiments will be conducted to determine if preincubation of the enzyme with Na^+ and oligomycin produces subsequent inhibition of the K^+-phosphatase. If Na^+-induced binding is of prime importance in oligomycin inhibition, then perhaps the K^+-phosphatase may not be a different enzyme but merely a portion of the $(Na^+ + K^+)$-ATPase.

Honors and Awards: None.

Publications: None.

Serial No. - NHI-148
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of action of drugs on sodium-dependent transport in the rat ileum

Previous Serial Number: NHI-325

Principal Investigator: Dr. Colin F. Chignell

Other Investigator: Mrs. Donnas K. Starkweather

Cooperating Unit: Dr. Chignell is a Research Associate in the Pharmacology-Toxicology Program.

Man Years:

Total:	0.4
Professional:	0.2
Other:	0.2

Project Description:

Objectives: The literature suggests that a number of purgative drugs may have a common site of action. Phenolphthalein and emodin block the active transport of sodium across the rabbit ileum and frog skin (Nature, 206: 1397, 1965). Phenolphthalein also inhibits the sodium-dependent uptake of 3-methyl-D-glucose by the hamster small intestine (Mol. Pharmacol., 3: 188, 1967) while both phenolphthalein and bisacodyl reduce intestinal glucose absorption in the rat (J. Pharm. Pharmacol., 19: 70, 1967). Microsomal $(Na^+ + K^+)$-ATPase, located in the brush border cells, appeared to be one possible site of action for these drugs since this enzyme is not only involved in active cation transport but also may be associated with the sodium-dependent active transport of other substrates (Ann. Rev. Physiol., 27: 415, 1965).

Methods Employed: Microsomes were prepared from the brush borders of rat small intestine by differential centrifugation of homogenates. ATPase activities were measured in the presence of optimal concentrations of Na^+, K^+, Mg^{++} and ATP. The $(Na^+ + K^+)$-ATPase activities were calculated by subtracting the activity obtained in the presence of Mg^{++} alone from that obtained with Mg^{++}, Na^+ and K^+.

Major Findings: All the purgative drugs tested inhibited the $(Na^+ + K^+)$-ATPase but not the Mg^{++}-ATPase activity of the intestinal microsomes (Table 1). Kinetic studies showed that inhibition by phenolphthalein is noncompetitive with respect to ATP, Na^+ and K^+ (Table 2). The inability of phenolphthalein

Table 1

Drug (10^{-4} M)	$(Na^+ + K^+)$-ATPase	Mg^{++}-ATPase
Control	20.7	30.8
Phenolphthalein disulfate	18.2	31.4
Phenolphthalein monoglucuronide	21.2	34.8
Danthron	7.0	33.4
Phlorizin	18.6	31.2
Phenol	21.0	31.0
Quinone	4.0	25.8
Hydroquinone	7.5	27.4
Control*	16.6	28.9
Phenolphthalein*	4.9	23.8
Bisacodyl*	9.9	27.1

*Activity measured in the presence of 1% ethanol.

disulfate and monoglucuronide to inhibit the enzyme suggested that the phenolic groups played an important role in inhibition. Phenol itself was inactive, although quinone and hydroquinone were good inhibitors. Since phenolphthalein, emodin, danthron and bisacodyl exist in the quinonoid form at physiological pH it appears that this is the biologically active form of the drug.

Table 2

Substrate	Enzyme	Vmax* Control	Vmax* Phenolphthalein (5×10^{-5} M)	Km (mM)	Ki† (mM)
ATP	Mg^{++}-ATPase	22.4	22.0	0.05	---
ATP	$(Na^+ + K^+)$-ATPase	28.8	15.1	0.087	0.06
KCl	"	34.9	21.2	3.5	0.08
NaCl	"	26.3	14.5	25.2	0.06

*μmoles/hr/mg protein
†for phenolphthalein

Significance: Inhibition of the process by which metabolic energy is made available for the transport of solutes from intestine to blood may be an important component of the action of a number of purgatives. At a more basic level the inhibition of $(Na^+ + K^+)$-ATPase by quinonoid forms of the drugs suggests the possible involvement of free radicals as reaction intermediates.

Proposed Course of Project: The possibility that free radical intermediates occur during the action of transport $(Na^+ + K^+)$-ATPase will be further investigated.

Honors and Awards: None.

Publications: None.

Serial No. - NHI-149
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Parasympathetic control of amylase levels in the rat submaxillary gland

Previous Serial No.: NHI-326

Principal Investigator: Dr. Colin F. Chignell

Other Investigators: Mrs. Loekie van de Ven
Mrs. Donnas K. Starkweather

Cooperating Unit: Dr. Chignell is a Research Associate in the Pharmacology-Toxicology Program, NIGMS
Mrs. van de Ven is a Guest Worker supported by the National Cystic Fibrosis Foundation.

Man Years: Total: 0.4
Professional: 0.2
Other: 0.2

Project Description:

Objectives: Schneyer and Schneyer have suggested (Ann. N.Y. Acad.Sci. 85: 189, 1960) that the increase in rat submaxillary gland amylase levels, observed after pilocarpine injection, is due to a stimulation of de novo enzyme synthesis. In a previous report, however, we showed that actinomycin D did not abolish the pilocarpine-stimulated increase in rat submaxillary gland amylase levels. Since actinomycin D is a potent inhibitor of de novo protein synthesis it appeared unlikely that pilocarpine could be stimulating the synthesis of amylase. This has now been confirmed by studying the effect of pilocarpine on the incorporation of amino acids into rat submaxillary gland amylase. The ability of protein synthesis inhibitor puromycin to prevent the pilocarpine-stimulated increase in rat submaxillary gland amylase has also been investigated.

Methods Employed: Male Long Evans rats (200 g) were starved (48 hrs), then injected with pilocarpine (2 mg, s.c.) every 30 min for 3 1/2 hr. Each animal was then injected with 4 μC of a mixture of uniformly labelled C^{14}-amino acids and sacrificed 20 min later. Submaxillary gland amylase was isolated and purified by absorption onto glycogen (B.B.A., 6a: 85, 1963). The purified enzyme was assayed for amylase activity (Methods in Enzymology, Vol. 1, p. 149), then dissolved in hyamine and counted in a liquid scintillation spectrometer.

401

Rats receiving puromycin were given an initial dose of 12 mg, followed by 6 mg every hour during the experiment. Injection of pilocarpine (2 mg, s.c.) was started one hour after the initial dose of puromycin and repeated every half hour. Four hours after the start of the pilocarpine injection the rats were sacrificed and their submaxillary glands removed, weighed and homogenized.

Major Findings: The effect of pilocarpine on the incorporation of amino acids into rat submaxillary gland amylase is shown in Table 1. The level of amylase in glands from pilocarpine-treated rats was seven times that found in the controls. Nevertheless, amylase isolated from the glands of pilocarpine-treated rats contained 45% fewer counts than amylase from control animals.

Table 1

Treatment	Amylase Activity (mg maltose/mg protein/15 min)		Radioactivity (c.p.m./mg protein)	
	Homogenate*	Purified Amylase†	Total gland protein†	Purified Amylase†
Control	1.58 ± 0.28	3340	176	160
Pilocarpine	11.20 ± 2.0	3600	152	90

*Each result is the mean from five animals ± standard error.

†These results were obtained with pooled homogenates from five animals.

Rats which had received puromycin still responded to pilocarpine by showing an increase in submaxillary gland amylase levels (Table 2).

Table 2

Treatment	Amylase Activity* (mg maltose/mg wet tissue/15 min)
Control	0.11 ± 0.018
Pilocarpine	2.92 ± 0.72
Pilocarpine + puromycin	4.19 ± 1.06
Puromycin	0.19 ± 0.015

*Each result is the mean from five animals ± standard error.

The data obtained with puromycin (Table 2) and actinomycin D (see previous report) are not consistent with the hypothesis that the pilocarpine

increases the de novo synthesis of submaxillary gland amylase. This conclusion is supported by the amino acid incorporation studies shown in Table 1.

Significance: The rat submaxillary gland offers a unique opportunity to study the mechanism whereby acetylcholine and other parasympathomimetic agents affect the level of a tissue enzyme. An understanding of such a system may enable us to interpret results obtained in other tissues in which enzymes are thought to be the target proteins for chemical transmitters.

Proposed Course of Project: The pilocarpine-stimulated decline in amino-acid incorporation into amylase could represent dilution by preformed enzyme present in a bound or inactive form. This possibility will be investigated. Since both synthesis and degradation play important roles in controlling the level of tissue enzymes the effect of pilocarpine on amylase catabolism will also be studied.

Honors and Awards: None.

Publications:

Chignell, C. F.: Mechanism of the pilocarpine-stimulated increase in rat submaxillary gland amylase levels. Biochem. Pharmacol. in press.

Serial No.- NHI-150
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The mechanism of sodium-dependent transport in rat ileum

Previous Serial Number: NHI-325

Principal Investigator: Dr. Elise Ann Brown

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.95
Professional:	0.95
Other:	0

Project Description:

Objectives: The active transport of glucose into erythrocytes, of catecholamines into nerve endings, and of amino acids or certain sugars across the small intestine are all strongly dependent on the presence of sodium ions. There is considerable evidence to suggest that sodium modifies affinity of some membrane component for these substrates, with a resultant facilitation of their diffusion. We are investigating the transport of alanine across the rat ileum in the hope that understanding of this mechanism will clarify the general problem of sodium-dependent transport.

The ultimate goal is the isolation from membranes of proteins which possess affinity for the transported components. Experiments in other laboratories indicate that agents with a specific affinity for glucose (which also is transported by a sodium-dependent mechanism) can be isolated from brush border cells of the small intestine. Proteins with specific affinities for transported substrates have also been isolated from bacterial membranes.

Methods Employed: The uptake of radioactive alanine-C^{14} has been measured as a function of sodium concentration using inverted intestinal sacs, intestinal strips, mucosal and serosal portions of intestinal strips, mucosal cell homogenates and brush border preparations. The concentration of alanine in tissue extracts was measured by determining total radioactivity or by the isolation of alanine as its N-dinitrophenyl derivative on thin layer chromatograms and determination of that portion of the radioactivity associated with the alanine spot.

404

Major Findings: Active transport of alanine across the rat ileum has a sodium-dependent component similar to that reported for rabbit and guinea pig gut. Efforts to regulate the level of this component by operations known to influence protein synthesis have been unsuccessful. Insulin did not affect alanine transport significantly even though it stimulates amino acid transport in muscle. Puromycin, which is known to affect sodium transport in toad bladder and to block the insulin-stimulated, sodium-dependent amino acid transport in kidney and diaphragm, was not useful because of the inactivation of control preparations during the required incubation times. Adrenalectomy had no effect on the maximal rate of alanine uptake attainable in the presence of sodium. There was, however, a statistically significant decrease in the rate of uptake in the absence of sodium.

Preliminary studies have been made of the filtration rates of substrates of varying molecular weight through a new high pressure, low porosity Amicon filter. The data indicate that changes in ultrafiltration rates provide a rapid and sensitive means for the estimation of the binding of small molecules to protein.

Significance to Biomedical Research and the Program of the Institute: The intestinal absorption of many nutrients, the uptake of glucose by red blood cells and the uptake mechanism which regulates the concentration of neurohumoral transmitters in the vicinity of sympathetic receptors are all transport processes regulated by sodium ions. Since the movement of these substrates across cell membranes is always in the direction of lower sodium concentration, the $(Na^+ + K^+)$ATPase which maintains a sodium gradient across the membrane provides the potential energy for the movement of these substances. The molecular mechanism by which sodium is coupled to the permeases, however, is completely unknown. It is commonly presumed that sodium confers added affinity for the transported substance on a membrane protein. Adsorption of the substrate could then cause a conformational change which could transfer the binding site to a region of lower sodium content. Isolation of a substance with affinity for the transported components would make possible a test of this hypothesis.

Proposed Course of Project: The capacity of brush border preparations and of proteins derived therefrom to bind alanine will be examined both in the presence and absence of sodium ion.

Honors and Awards: None.

Publications: None.

Serial No. - NHI-151
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Formation and metabolism of neurohumoral transmitter substances in marine animals

Previous Serial Number: NHI-367

Principal Investigator: Dr. Elise Ann Brown

Others Investigators: None

Cooperating Unit: Dr. E. G. Trams, Laboratory of Neurochemistry, National Institute of Neurological Diseases and Blindness

Man Years: Total: 0.05.

Project Description:

Objectives: Studies of the metabolism of appropriate compounds in more primitive animals can offer clues to the evolutionary development of those enzyme systems which break down both foreign compounds and naturally occurring substances in the body. Levels of the classically studied drug metabolizing enzymes have therefore been determined in several tissues of pelagic animals. The observation that shark interrenal body contained surprisingly high levels of an endogenous substrate for transmethylating enzymes led to the discovery that conversion of norepinephrine to epinephrine takes place in this organ. The elasmobranch interrenal body is analogous to the cortex of the mammalian adrenal gland. The presence of what is usually a medullary function in such a tissue suggests that this organ can be utilized to study the interrelationship between corticosteroids and catecholamines.

Methods: Enzymes of catecholamine metabolism were studied in vitro in fresh or frozen tissues by standard techniques. Radioactive precursors were used as substrates where indicated. Isotopic catecholamines, or derivatives, were administered to animals and the distribution and metabolism was followed by standard sampling techniques.

Major Findings: Only very low activity of a number of common detoxication reactions could be measured in shark liver microsomal preparations for the study. Compared to most mammalian systems, azoreductase and O-demethylation activities were low. The yield of microsomal protein from shark liver was also quite low (3.7 mg/g) in comparison to the rat (26 mg/g). The activity of these shark enzymes, if related to microsomal protein, is about 10% that of the rat enzymes. The contribution of such low levels of activity,

however, may not be insignificant in the overall homeostatic mechanism of this species of fish because potential substrates presumably are encountered at low concentrations only. Neither azoreductase activity nor nitroreductase activity was observed with a tiger shark (Galeocerdo cuvieri) liver enzyme preparation. Hydroxylation reactions, studied with 3,4-benzpyrene or antipyrine as substrate were absent in a tiger shark, a dusky shark (Carcharhinus obscurus) and a brown shark (Carcharhinus milberti).

Catechol-O-methyltransferase (COMT) activity was found to be present at various levels in all the shark tissues studied here. The highest levels were observed in the kidneys (mesonephros) of adult sharks. Levels of COMT in the kidney of a brown shark fetus were quite low in contrast. In the elasmobranch it appears that this enzyme is principally associated with the microsomal fraction although some activity was observed in soluble fractions prepared from kidney homogenates. Magnesium ions were not required as they are for the mammalian enzyme.

Optimum activity with Squalus acanthias (spiny dogfish) COMT was obtained at 37°C but an incubation temperature of 25°C was chosen for most of the experiments since this was thought to be more representative of the temperature of the natural environment. With norepinephrine as substrate the methyl transferase activity of both kidney and liver was strongly inhibited by pyrogallol or tropolone. N-methyltransferase and monophenol-O-methyltransferase activities were insignificant. The methyltransferase system, therefore, consisted principally of COMT. Monoamine oxidase activity was low with respect to that of COMT.

Interrenal body is analogous to the adrenal cortex found in more advanced animals. In elasmobranchs, the interrenal body is anatomically separate from the intrarenal tissue (adrenal medullary counterpart). Assays for catechol-O-methyltransferase and phenylethanolamine-N-methyltransferase with S-adenosylmethionine-C^{14} showed the presence of both enzymes in homogenates of interrenal bodies obtained from several shark species and a sting ray. Evidence for an endogenous acceptor of the labeled methyl suggested the presence of catechol amines, and large amounts of both epinephrine and norepinephrine were indeed found in the interrenal body. This organ may be a major source of epinephrine in the elasmobranch.

This hypothesis was further strengthened by studying the distribution of norepinephrine-7-H^3 in nurse sharks. Interrenal body, next to kidney, was the most active tissue in concentrating the labeled catechol. Conversion of norepinephrine-H^3 to its N-methylated product had occurred and both catechols appeared to be metabolized principally by O-methylation.

The finding that the catechol-O-methyltransferase system in shark liver is a particulate rather than a soluble enzyme is of interest. Microsomal enzymes have been considered to be comparatively primitive (undifferentiated) from a phylogenetic point of view. A study of the development of this enzyme through a number of species which represent different stages of evolution might further our understanding of the evolution of biochemical processes and

their macromolecular catalysts.

Significance to Biomedical Research and the Program of the Institute: Certain pelagic species, as the elasmobranchs, have not evolved significantly since mesozoic times. Thus, some insight may be gained into the evolution of some biochemical processes from their primitive forms. It has been suggested that the juxtaposition of adrenal cortex and medulla in higher animals is an evolutionary adaptation to facilitate control of epinephrine synthesis by cortical steroids. The presence of an epinephrine synthetic system in a supposedly cortical tissue suggests that the interrelationship between corticosteroids and catecholamines may be studied in the interrenal body.

Proposed Course of Project: Studies will be made of the physiology and biochemistry of the elasmobranch interrenal body. Studies will be undertaken on the enzymes which are involved in catecholamine biosynthesis and metabolism. Pilot studies will be concerned with the identity of the major steroids in the interrenal body. Studies of the role of steroid hormones in the regulation of enzyme synthesis in this tissue will be carried out.

Honors and Awards: None.

Publications:

Trams, Eberhard G. and Brown, Elise A. B.: Metabolic alterations of catecholamines and other compounds in elasmobranch tissues. Proc. Soc. Exp. Biol. 125: 253-256 (1967).

Lauter, C. J., Brown, E.A.B. and Trams, E. G.: Composition of plasma lipoproteins of the spiny dogfish Squalus acanthias. Comp. Biochem. Physiol. 24: 243-247 (1968).

Brown, E.A.B. and Trams, E.G.: Catecholamine metabolism in elasmobranch interrenal body. Comp. Biochem. Physiol. 24: in press (1968).

Serial No.- NHI-152
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the molecular nature of adrenergic receptors. Alkylation of alpha adrenergic receptors in mouse spleen

Previous Serial Number: NHI-327

Principal Investigators: Dr. Louis J. Ignarro
Dr. Elwood O. Titus

Other Investigators: None

Cooperating Units: Dr. Ignarro is an NIH Postdoctoral Fellow, NHI

Man Years:

Total:	0.9
Professional:	0.9
Other:	0

Project Description:

Objectives: The ultimate goal of this project is to isolate from sympathetically innervated tissue the receptor molecules with which norepinephrin interacts in eliciting a physiological response. Irreversible alkylation by isotopically labeled inhibitors offers the possibility of introducing into the receptor a radioactive label that can be used to guide the subsequent isolatio of cellular components associated with the active site.

Since it is important to establish that labelling is in fact accompanied by inhibition of the receptor, reactions with antagonists are conducted with isolated organs in which the pharmacological effects of the inhibitor can be observed. Studies described in previous reports indicate that the isolated mouse spleen is an appropriate organ for the labeling studies.

Methods Employed: Isotonic contraction and relaxation of the isolated mouse spleen suspended in physiological saline are recorded. The effects of sympatholytic and sympathomimetic drugs are recorded as a function of dosage. Measurements of the uptake of radioactive drugs into tissue are made by conven tional counting procedures after digestion of tissue homogenates.

Major Findings: Previous studies have established the presence in isolated mouse spleen of both the expected alpha adrenergic receptors and beta receptors that initiate splenic relaxation. Blockade of the latter was found

409

to permit quantitative studies of the alpha contractile response in the usual manner. More recent studies have dealt with: 1) Analysis of the quantitative relationship between splenic contraction and the concentration of a series of norepinephrine analogs. 2) The effects on these relationships of alkylation of the alpha receptor. 3) The ability of norepinephrine analogues of differing structure to protect the receptor against alkylation.

1) Concentration-response relationships: Splenic contraction may be related to the concentration of norepinephrine by the classical Michaelis-Menton relationship, suggesting that the contraction is directly proportional to the percentage of receptors occupied. This relationship, however, does not hold for any N-substituted derivatives of norepinephrine. The form of the concentration curves suggests that N-substituents may extend into an area adjacent to the receptor and that occupation of this area decreases the affinity of the receptor proper for the catecholamine nucleus.

2) The effects of receptor alkylation: N,N-dimethyl-2-bromo-2-[4'-methylphenyl] ethylamine (L-35) is an alkylating agent which exhibits several thousand times the potency of dibenamine as an alpha adrenergic inhibitor. Spleens temporarily exposed to L-35 in concentrations up to 3×10^{-8} M exhibit changes in the contractile response to norepinephrine that can be accounted for quantitatively in terms of a decreased association constant for the interaction of norepinephrine with the receptor. Concentrations of L-35 greater than 10^{-8} M cause a qualitatively different response in which the maximal attainable contraction is reduced.

If analogues of norepinephrine containing N-substituents larger than methyl are used to test the effects of L-35, only the latter type of response is seen, irrespective of the concentrations of L-35.

3) Protection experiments: If norepinephrine or its N-substitute analogues are present during interaction of isolated spleens with L-35, the alpha-blockade which is usually apparent after washing away of the excess L-35 is reduced. Since norepinephrine is less than 20% as effective as its N-substituted derivatives, it appears that N-substitution is required for effective protection of the site of alkylation.

Taken in sum the above experiments suggest that adjacent to the receptor site which accepts the catecholamine nucleus there exists an area, whose occupancy by alkyl substituents can allosterically modify the affinity of the receptor. Occupation of this area as a consequence of irreversible alkylation could account for at least part of the effects of L-35.

4) Kinetic studies of recovery from alpha blockade: Although the effects of L-35 are irreversible in the sense that covalent bond formation is involved, the duration of alkylation is only a few hours owing to an intramolecularly assisted hydrolytic mechanism by which the bond to the receptor is broken. Preliminary measurements of the rate of recovery of responsiveness to catecholamines and of the rate of removal of radioactivity from spleens that have been labeled with tritiated L-35 indicate that both are first order processes

with half-lives of the order of 33 to 39 minutes. Some variations in the rate of recovery of responsiveness to catecholamines have been encountered when the concentrations of amine used in the testing process were varied. These anomalies require further experimentation.

5) Uptake of L-35-H^3 in the isolated mouse spleen: L-35-H^3 reacts with two major components of the spleen, one of which subsequently releases L-35-H^3 by a first order process with a half-time of about 33 min. The second component of binding holds onto the L-35-H^3 very tightly.

A reduction in temperature of incubation from 37°C to 25°C results in a reduction of 1) the amount of L-35-H^3 taken up, 2) the amount of releaseable L-35-H^3 and 3) the amount of tightly bound L-35-H^3. The rate of release of L-35-H^3 is unaffected by such a temperature reduction.

Iodoacetate (10^{-3} M) or 2,4-dinitrophenol (10^{-4} M) do not inhibit uptake of L-35-H^3. Cocaine (3 x 10^{-4} M) and reserpine (10^{-4} M) inhibit uptake of L-35-H^3 by 24-28% and 26-41% respectively. Isoproterenol also blocks L-35-H^3 uptake by about 29%. Reserpinization of mice (5 mg/kg for 12 hr) did not affect the uptake of L-35-H^3 by the spleen.

Reserpinization and immunosympathectomy appear to reduce the amount of tightly bound L-35-H^3 without affecting the amount of releaseable L-35-H^3. The rates of splenic release of L-35-H^3 from reserpinized and immunosympathectomized animals appeared to increase slightly to half-times of 27 min and 21 min respectively.

Significance to Biomedical Research and the Program of the Institute: Essentially nothing is known of the structures with which sympathomimetic and sympatholytic agents interact in exerting control of cardiovascular phenomena. Ultimately a knowledge of the topology of receptor surfaces should provide a more rational basis for drug design.

Proposed Course of Project: Radioactively labeled L-35 of higher specific activity than now available will be used to label the receptor site (or the adjacent regulatory area postulated in this report). Studies on the labeling of presynaptic uptake sites for catecholamines will also be undertaken.

Honors and Awards: None.

Publications:

Ignarro, Louis J. and Titus, Elwood: The presence of antagonistically acting alpha and beta adrenergic receptors in the mouse spleen. J. Pharmacol. Exp. Therap. 160: 72-80, 1968.

411

Serial No. NHI-153
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Rate of Synthesis of Adipose Tissue Norepinephrine Under Conditions Associated With Increased Release of FFA and Glycerol

Previous Serial Number: None

Principal Investigators: Dr. Gian L. Gessa
Dr. George A. Clay

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.2
Professional:	0.2
Other:	0

Project Description:

Objectives: The presence of NE in adipose tissue has been well-demonstrated. NE is contained in the sympathetic fibers and disappears after surgical denervation. Since both NE and the sympathetic stimulation induce release of FFA and glycerol and these effects are blocked by adrenergic blocking agents, it is reasonable to conclude that this metabolic change is mediated by release of NE by nerve ending. Since a number of polypeptides also releases FFA, however, it is still possible that the rapid mobilization of FFA is evoked physiologically by hormones other than catecholamines. In addition the relative role played by the catecholamines released by sympathetic nerves and adrenal medulla still remain obscure.

Studies of the synthesis rate of NE give a better indication of the sympathetic tone than the levels of the amine. These might remain constant or even decline after an increased rate of release. Therefore, we have studied the synthesis rate of NE in adipose tissue under basal conditions and under different conditions associated with enhanced release of FFA to determine the role played by the sympathetic nervous system.

Methods Employed: Male Sprague-Dawley rats weighing 180-200 g were used. d,l-α-Methyl-p-tyrosine (d-MT) was dissolved in H_2O at pH 8.5 and injected i.v. at the doses and times that will be indicated in the results.

412

In some experiments the methyl ester of α-MT was used. The animals were sacrified by cervical fracture, and organs were frozen until assayed for NE. The NE content was extracted onto alumina with a modification of the method described by Brodie et al. (Brodie, B. B., J. Pharmacol. Exp. Ther. 152: 340, 1966) and the extracted catecholamines were oxidized by the procedure described by Chang, (Chang, C. C., Int. J. Neuropharmacol. 3: 643, 1964).

Accordingly, tissues are homogenized into 4-8 volumes of 0.4 N perchloric acid. The homogenate is filtered with paper filter. An aliquot of the glass filtrate (10 ml for epididymal fat pad) is diluted with 2.5 M Tris, pH 10 (1:1.5) and the catecholamines adsorbed onto alumina. They are then eluted from alumina with 0.2 N acetic acid and oxidized.

Major Findings: The normal level of NE in interscapular brown fat 0.87 is about μg/g of wet tissues and is thus not much lower than that of heart. The level in epididymal fat is about 0.12 μg/g. After administration of α-MT the NE levels declined with half-life of 10 hours for the heart and 6 hours for brown and white adipose tissue. Because of the low starting levels in the epididymal fat, the values could not be determined with precision.

From the half-life of the decline of NE after administration of and from its steady-state levels in control animals we have calculated the synthesis rates in brown and white fat and compared them with that of heart: i.e. the μg of NE synthesized per g of tissue and per hour. The synthesis rate of brown fat is about 50% lower than that of heart; that of white is 1/5 that of heart.

The rate of NE depletion in adipose tissue after blockade of synthesis was studied under conditions which evoke an increased release of FFA and glycerol.

Swimming at 20°C, a stressful condition where there is a combined effect of low temperature and muscular exercise, decreased the NE levels in adipose tissue in animals whose NE synthesis is not blocked. The combination of swimming with α-MT produces a much greater depletion of NE (both in brown and white fat) than in each treatment alone.

Table 1 shows the influence of acute and chronic exposure to cold on the NE depletion obtained with α-MT.

Both warm and cold-adapted animals show a far greater response to the inhibition of catecholamine synthesis when exposed to cold. In cold-adapted animals, the norepinephrine concentration in the interscapular brown fat is about 80% higher than in warm-adapted ones.

Immobilization stress is known to increase FFA release and was shown by Corrodi et al. (Corrodi, H. et al. Life Sci. 7: 107, 1968) to the depletion of NE in brain after inhibition of NE synthesis. The rats were

TABLE I

EFFECT OF COLD ON THE LEVELS OF NE IN THE INTERSCAPULAR FAT PAD AFTER INHIBITION OF TYROSINE HYDROXYLASE

Acclimatized for 3 weeks to:	Exposed to:	Levels of NE µg/g hrs after α-MT 0	6
21° C	21° C	0.87 ± 0.08	0.60 ± 0.08
21° C	3° C		0.11 ± 0.04
3° C	3° C	1.32 ± 0.11	0.11 ± 0.06

200 and 100 µg/kg of α-MT were given i.v. at 0 and 3 hr, respectively. Each value is an average (± S.E.) from at least 5 experiments.

immobilized by wrapping them in a wire net, which does not allow them any movement.

Stress by restraint alone did not change the levels of NE in the adipose tissue, but caused a greater depletion of NE after inhibition of its synthesis. The role of sympathetic system in the increased FFA release by fasting is debatable. We have found that there is no greater depletion in faster animals than in fed ones.

Significance to Bio-medical Research and the Program of the Institute: All examined conditions, except fasting, associated with an increased release in FFA and glycerol accelerate the NE depletion in adipose tissue obtained with a tyrosine hydroxylase inhibitor.

Since the degree of amine depletion obtained with synthesis inhibitors is highly dependent on the nervous impulse flow, these results indicate that the sympathetic fibers to the adipose tissue are activated in all conditions associated with an increase in FFA release. These studies provide a better understanding of the role of NE in adipose tissue.

Proposed Course of Project: Questions of immediate importance will be to study the rate of synthesis of NE in the adipose tissue in many dismetabolic situations, like experimental diabetes, hypo- and hyperthyroidism, various obesities, which are associated with increased release of FFA.

Honors and Awards: None

Publications: None

Serial No. NHI-154
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Role of Norepinephrine in Adipose Tissue: Depletion of Norepinephrine by Amphetamine

Previous Serial Number: None

Principal Investigators: Dr. George A. Clay
Dr. Gian L. Gessa

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.5
Professional:	0.5
Other:	0.0

Project Description:

Objectives: Amphetamine is known to exert an antiappetite effect and an elevation of free fatty acids (FFA's) in the bloodstream. Since the mobilization of FFA's is thought to be mediated by the sympathetic nervous system, we have studied the effect of amphetamine upon the peripheral stores of NE in the intrascapular and epididymal fat pads of rats in order to further understand the role of the sympathetic nervous system in adipose tissue.

Major Findings: (1) Depletion of NE with amphetamine. A single dose of 5 mg/kg i.p. of amphetamine produced significant depletion of NE stores in both the intrascapular brown fat pad and the epididymal white fat pad, but at an entirely different rate than was found in heart. Heart NE levels were unchanged during the first hours following administration of the drug, then slowly declined to about 50% of control levels at 24 hours. Intrascapular brown fat NE levels declined rapidly to about 60% of control levels during the first hour, were 50% of initial levels at 6 hours, and had returned to normal levels by 24 hours. Epididymal fat pad levels followed a similar pattern of depletion to that of brown fat, with a level of 75% depletion at 6 hours and a return to normal at 24 hours.

Specificity of NE depletion in adipose tissue by amphetamine: Rapid depletion of the adipose tissue NE was not a general response to sympathomimetic amines. Cocaine in single or multiple doses did not change the levels of NE in heart or adipose tissue. Metaraminol depleted NE in heart,

416

brown fat and white fat at equal rates.

Significance to Bio-medical Research and the Program of the Institute: Amphetamine caused a rapid depletion of NE in adipose tissue compared with a long slow decline of NE store in the heart. This rapid release of adipose tissue NE appears to be specific for amphetamine.

Proposed Course of Project: Further study of the role of the sympathetic nervous system in adipose tissue is planned. Turnover of NE under various conditions including administration of amphetamine will be studied in these tissues.

Honors and Awards: None

Publications: None

Serial No. -NHI-155
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effect of Ions on NE-Induced Lipolysis and NE Uptake into Localized Fat Cells

Previous Serial Number: None

Principal Investigators: Dr. Claus-Jurgen Estler and Dr. Gopal Krishna

Other Investigators: None

Cooperating Units: Dr. Estler was a guest worker paid by the Medizinisch Pharmazeutische Studiengesellschaft, Frankfurt, Germany

Man Years:

Total:	0.8
Professional:	0.8
Other:	0.0

Project Description:

Objectives: Previous studies from this laboratory have shown that in adrenalectomized rats catecholamines and glucagon failed to exhibit a lipolytic effect and that lipolysis could be restored either by administration of glucocorticoids or mineralocoid hormones. *In vitro* experiments showed that this effect could not be ascribed to the action of the hormones themselves. It was postulated that the lack of lipolytic response in adrenalectomized animals might be due to an electrolyte imbalance and especially to changes in the electrolyte concentration near the adrenergic receptor sites in the adipose tissue.

In order to test this hypothesis, the effect of electrolytes on the norepinephrine-induced lipolysis in isolated fat cells were examined. Fat cells were chosen as a model for the experiments, since the changes in the electrolyte concentrations of the incubation media would affect the receptor sites more instantaneously than in whole adipose tissue.

Methods Employed: Isolated fat cells were prepared from rats' epididymal adipose tissue, according to a modification of the method of Rodbell. After isolation they were incubated at 37°C in Krebs-Ringer phosphate buffer or various other media from which one or more of the anions or cations normally present in Krebs-Ringer phosphate buffer had been omitted. Each medium contained 5% bovine albumin. The pH of the medium was adjusted to 7.4 with hydrochloric acid and lithium hydroxide which did not show any influence on lipolysis and uptake of norepinephrine. Norepinephrine was added to the media

418

to give a final concentration of 10^{-6} M. The rate of lipolysis was estimated by measuring the release of free fatty acids into the medium after 0, 10, 20, and 40 minutes incubation. The assay of free fatty acids was carried out according to a modification of the method of Novak.

Major Findings: When fat cells were incubated in Krebs-Ringer phosphate buffer free fatty acids were released into the medium at a rate which was constant for at least 40 minutes. This rate was greatly reduced, if the cells were incubated in an electrolyte-free medium, for instance in isotonic sucrose solution instead of Krebs-Ringer phosphate buffer. To determine which of the ions present in Krebs-Ringer phosphate buffer are the most important to maintain lipolysis, electrolytes were omitted from Krebs-Ringer phosphate buffer and fat cells were incubated in those media.

Replacement of sodium in Krebs-Ringer phosphate buffer by sucrose greatly impaired lipolysis. The rate was only 37% of that observed in Krebs-Ringer phosphate buffer. Potassium seems to be less important than sodium; when potassium was omitted, the rate of lipolysis slowed down only in the second hal of the experiment. This effect seems to be dependent on the norepinephrine concentration of the medium. As Ho and coworkers have reported recently, lipolysis can be reduced by about 80% in potassium-free media with lower concentrations of norepinephrine present.

Omission of magnesium did not affect the rate of lipolysis, and therefore seems not to be an essential ion for lipolysis. On the other hand, when calciu was omitted, lipolysis is markedly reduced. Results similar to those shown wit calcium-free media could be observed when phosphate was omitted from the medium and replaced by sucrose or chloride. Thus it appears that sodium, potassium, calcium and phosphate are required to maintain norepinephrine-induced lipolysis at an optimal rate.

Since it was felt that the failure of the lipolytic response could be due to an impaired uptake of norepinephrine into the fat cells, we also studied the effect of electrolytes on the uptake of norepinephrine into isolated fat cells. For these studies tritium-labeled norepinephrine was mixed with carried norepinephrine and added to the various incubation media at a final concentratio of 10^{-6} M and 0.4 μc/ml. After 0, 10 and 20 minutes incubation the fat cells were separated from the media. The radioactivity in the separated cells was counted in a liquid scintillation counter. Corrections for the extracellular medium still in contact with the cells after their separation were made by using ^{14}C-labeled inulin as an extracellular marker.

The uptake of norepinephrine is expressed as the ratio of concentrations of tritium-labeled norepinephrine in the aqueous phase of the cells versus outside the cells. For this purpose the water space of the cells had to be determined in separate experiments by using tritiated water and C^{14}-labeled inulin as an extracellular marker. It was found to be 7% of the cell volume, and this value was used for the calculations.

Fat cells incubated in Krebs-Ringer phosphate buffer take up norepinephrine by a process against a concentration gradient. With 10^{-6} M norepinephrine in the medium half the steady state level can be obtained after about two minutes. At a steady state the ratio of norepinephrine in the cell water versus norepinephrine in the medium is about 4. This ratio was further increased at lower norepinephrine concentrations in the medium.

If fat cells are incubated without electrolytes in isotonic sucrose solution the uptake of norepinephrine is greatly reduced in comparison to cells incubated in Krebs-Ringer phosphate buffer. The ratio of norepinephrine inside the cells versus outside approaches only unity within 20 minutes.

To check which ions are the most important for the uptake process, electrolytes were omitted stepwise from Krebs-Ringer phosphate buffer. Omission of sodium, which inhibits lipolysis almost completely, has no effect on the uptake of norepinephrine. After 20 minutes incubation, the ratio of norepinephrine inside the cells versus outside is about the same as in Krebs-Ringer phosphate buffer. Removal of potassium also does not affect the uptake of norepinephrine to any great extent.

On the other hand, omission of magnesium, which has no effect on the rate of lipolysis, lowers the uptake of norepinephrine markedly. Calcium and phosphate, which have some effect on lipolysis, seem also to be important for the uptake of norepinephrine. When fat cells are incubated in media free from calcium, magnesium, or phosphate, the uptake of norepinephrine is much slower, and the ratio of norepinephrine inside the cells versus outside approaches only values of about 1-1.5 within 20 minutes.

Although it seems that calcium, phosphate, and magnesium are the most important ions required for the uptake of norepinephrine from the medium into the cells, none of these ions alone added to a sucrose solution could restore the uptake process to normal. If fat cells are incubated in sucrose solution and calcium or phosphate are added in concentrations of 16 or 18 mM respectively--these are the concentrations at which these ions are present in Krebs-Ringer phosphate buffer--the uptake is not significantly enhanced. The same is true for sodium. Fat cells incubated in isotonic saline take up norepinephrine to a ratio of about 1.5.

Significance to Bio-medical Research and the Program of the Institute:
It is difficult to define the significance of all of our results. The finding that potassium and above all sodium are required to maintain the lipolytic effect of norepinephrine at a normal rate might explain--at least in part--the reduced response to norepinephrine in adrenalectomized animals. This, however, does not seem to be due to an impaired uptake of norepinephrine into the cells, because the uptake of norepinephrine is not dependent on sodium and potassium, at least not at norepinephrine concentrations of 10^{-6} M in the medium. It appears that at this concentration of norepinephrine there is no apparent correlation between the amount of norepinephrine taken up by the cells and their lipolytic response. It must be borne in mind however, that

norepinephrine concentrations of 10^{-6} M are well above the physiological concentrations of norepinephrine, ant it might be conceivable that at much lower concentrations the uptake process might play an important role or become rate limiting for the effectiveness of norepinephrine in eliciting a lipolytic response. Further studies are necessary to answer these questions.

Proposed Course of Project: Further attempts will be made to understand the mechanisms of uptake of NE into fat cells and its significance to NE-induced lipolysis by use of specific metabolic inhibition.

Honors and Awards: None

Publications: None

421

Serial No. NHI- 156
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: In Vitro Induction of Adenyl Cyclase by Tri-Iodo-Thyronine in Isolated Fat Cells

Previous Serial Number: None

Principal Investigators: Dr. Henry Bourne
Dr. Gopal Krishna

Other Investigators: Dr. George L. Pauk

Cooperating Units: Dr. Bourne is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Man Years:

Total:	0.1
Professional:	0.1
Other:	0.0

Project Description:

Objectives: Previous studies in this laboratory (Krishna et al.) have shown that thyroid hormones administered to rats in vivo are able to increase markedly the lipolytic response of isolated fat cells to norepinephrine, and that this increase is due to an increase in the amount of adenyl cyclase in these cells. These experiments attempted to elucidate the mechanism of this effect of the thyroid hormones on adenyl cyclase (AC).

Methods Employed: Fat cells were isolated by the method of Rodbell. Adenyl cyclase was measured by the method of Krishna.

Major Findings: Initial experiments showed that incubation of isolated fat cells for 2 hours with tri-iodo-thyronine, 10^{-6}M in vitro produced a 50% increase in adenyl cyclase, and that this effect on AC was blocked by puromycin. However, this result could not be repeated, and an exhaustive attempt at finding the reason for this failure was unsuccessful. In preliminary experiments measurement of cyclic adenosine 3',5'-monophosphate levels in identical incubations have not shown a significant difference between T_3-treated and control cells.

Significance to Bio-medical Research and the Program of the Institute: Such studies will eventually prove important for understanding the role of adenyl cyclase in adipose tissue, as well as the mechanism of action of thyroid hormones.

422

Proposed Course of Project: The *in vitro* experiments will be continued, with a view to seeing what variables are responsible for the inability to reproduce the results.

Honors and Awards: None

Publications: None

Serial No. NHI-157
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effects of Cortisone Treatment in vivo on the Norepinephrine-Stimulated Lipolysis of Fat Cells in vitro

Previous Serial Number: None

Principal Investigators: Dr. Ian F. Skidmore
Dr. Henry R. Bourne
Dr. Gopal Krishna

Other Investigators: Dr. George L. Pauk

Cooperating Units: Dr. Bourne is a Research Associate in Pharmacology Toxicology Program, NIGMS

Man Years:

Total:	0.6
Professional:	0.6
Other:	0

Project Description:

Objectives: Studies have shown that for a satisfactory lipolytic response to norepinephrine in vivo the animal requires an intact active adrenal cortex. Stimulation of fatty acid release by norepinephrine both in vivo and in vitro is thought to be mediated through the formation of cyclic 3'5' AMP within the cells and maximal rates of lipolysis are only obtained when theophylline is present to inhibit the cyclic 3'5'AMP phosphodiesterase present within the cells. These experiments were designed to investigate the "permissive" effect of corticosteroids by showing whether the administration of cortisone to normal animals could increase the norepinephrine-stimulated lipolysis in adipose tissue cells, and to investigate methods by which this increase could occur.

Methods Employed: Fat cells were isolated by the method of Rodbell from the epididymal fat pads of fed rats weighing 115 to 140 grams. Cells from normal and cortisone-treated animals were incubated in Krebs-Ringer phosphate buffer containing 5% albumin in the presence of varying concentrations of norepinephrine. Lipolysis was estimated by measuring the amount of glycerol liberated from a suspension of cells in 15 or 20 minutes at 37°C. Glycerol was converted to formaldehyde and measured chemically by the method of Nash. Dose response curves were constructed to determine the

sensitivity of the cells to norepinephrine and the maximal obtained rates of lipolysis were measured in the presence and absence of theophylline. Rates of lipolysis were expressed in terms of the amount of DNA present in the preparation. Adenyl cyclase and phosphodiesterase were measured by the methods of Krishna et al.

Major Findings: (1) The sensitivity of the lipolysis to norepinephrine (as measured by the concentration of norepinephrine required to produce 50% of maximal lipolysis in the absence of theophylline) was increased slightly by the administration of a single dose of cortisone acetate (25 or 100 mg/kg, s.c.) given four hours before sacrifice. This increase in sensitivity was not increased further if the cortisone was given six hours before sacrifice.

(2) The maximal rate of lipolysis was increased by treatment of the animals with cortisone. The increase was apparent after three to four hours and the percentage increase appeared to be the same in the presence and absence of theophylline, indicating that the total amount of lipase may be increased by cortisone treatment in vivo.

(3) Cortisone treatment does not increase fat cell phosphodiesterase or adenyl cyclase (measured in the presence of NaF). In addition, preliminary experiments showed that the sensitivity of the adenyl cyclase to norepinephrine is also not increased.

(4) The lipolytic response of isolated adipose tissue cells to theophylline is markedly increased by pretreatment of the animals with cortisone.

Since theophylline is thought to cause lipolysis by blocking the intracellular degradation of 3',5'-cyclic adenosine monophosphate by phosphodiesterase, an increase in lipolytic response to theophylline could indicate either a higher rate of formation of cyclic AMP or an increased activity of the lipase enzyme. That the latter is probably the case is suggested by experiments with dibutyryl cyclic AMP. This substance is an analog of the endogenous cyclic nucleotide which is able to activate lipase by a mechanism presumably similar to that of cyclic AMP itself. In preliminary experiments dibutyryl cyclic AMP caused considerably more lipolysis in fat cells from cortisone-treated rats than in controls, suggesting that it is the lipase system which is most markedly affected by cortisone. Preliminary experiments of measurement of cyclic AMP in isolated fat cells have shown no cortisone effect on basal levels or response to norepinephrine.

Significance to Bio-medical Research and the Program of the Institute: Elucidation of the effect of hormones, such as cortisone, on lipolysis by adipose tissue will be an important contribution to an understanding of their general effects in many other tissues.

425

Proposed Course of Study: These studies are to be extended to include the effects of chronic treatment with corticosteroids and the effects of adrenalectomy and the administration of cortisone to adrenalectomized animals on the norepinephrine stimulated lipolysis.

Honors and Awards: None

Publications: None

426

Serial No. NHI-158
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Role of Cyclic 3',5'-AMP as a Mediator of Hormones in Brain

Previous Serial Number: None

Principal Investigators: Dr. Gopal Krishna
Dr. Gian L. Gessa

Other Investigators: Dr. Bruce R. Ditzion

Cooperating Units: Dr. Ditzion is a Research Associate in the Pharmacology-Toxicology Training Program, NIGMS

Man Years:

Total:	1.0
Professional:	1.0
Other:	0.0

Project Description:

Objectives: Previous reports from this laboratory have indicated that the enzyme systems involved in the synthesis and degradation of cyclic 3',5'-AMP are widely distributed in various areas of brain. Moreover, NE and dopamine could activate in vitro the enzyme adenyl cyclase, which is involved in the synthesis of cyclic 3',5'-AMP. The main objective of the present study is to understand the functions mediated by cyclic 3',5'-AMP in the brain by injecting this compound or its dibutyryl derivative into various discrete areas of brain and observe the behavioral changes thus produced. Cats, rats and rabbits were used in these experiments.

Methods Employed: Cannulas were chronically implanted into various discrete areas of brain using standard stereotaxic techniques. Cats and rats were used for such studies. The animals were left to recuperate after the operations for at least 2-3 days. The compounds were injected in 1-10 µl volumes. When the compounds were injected intracisternally, the animals were anesthetized lightly with ether.

Major Findings: The enzyme phosphodiesterase is distributed widely in brain. This enzyme converts cyclic 3',5'-AMP to 5'-AMP, thus terminating its action within minutes after injection. In contrast the dibutytyl derivative of cyclic 3',5'-AMP is hydrolyzed very slowly in the brain and

thus its action persists for a longer time. For this reason, dibutyryl derivative of cyclic AMP was used throughout this study.

In order to show that the effects are specific for cyclic 3',5'-AMP and not for adenine nucleotide, we injected dibutyryl 5'-AMP as a control.

After injection of dibutyryl cyclic 3',5'-AMP (100 μg) into lateral ventricle, rats displayed an intense hyperactivity which cannot be observed with any other stimulant. The animals went into convulsion 20 minutes after treatment. This effect was not prevented by pretreatment of the animals with reserpine or chlorpromazine--on the contrary, potentiation of the hyperactivity occurred. Similar results were obtained when dibutyryl cyclic 3',5'-AMP was injected into hypothalamus of rats.

The injection of dibutyryl cyclic 3',5'-AMP (200-500 μg) into hypothalamus of conscious cats produced an intense ergotropic stimulation characterized by hyperactivity, sham rage, mydriasis, salivation, hyperthermia, and hallucinatory behavior. After injection of the higher doses, the animals showed recurrent convulsions and eventually died in hyperthermia (43°C).

When this compound was injected into "reticular formation" of conscious cat, it caused a marked catatonia which lasted for hours: when cats were placed in different unnatural positions, they would retain the positions for a very long time.

When the animals were injected with cyclic 3',5'-GMP (1000 μg) into hypothalamus, sedation occurred within 4 minutes after injection, followed by miosis, sleep with loss of righting reflex. When the same animals, after recovery from the effect of cyclic 3',5'-GMP, were injected with dibutyryl cyclic 3',5'-AMP, they showed the described excitatory syndrome.

None of these effects were produced when injected with 5'-AMP or its dibutyryl derivative.

Significance to Bio-medical Research and the Program of the Institute: Microinjection of dibutyryl cyclic 3',5'-AMP in various discrete areas of the brain, where cyclic 3',5'-AMP is normally present, would allow not only the understanding of the role played by cyclic 3',5'-AMP in mediating certain functions of neurohormones but also the understanding of the functions of certain areas of the brain.

Proposed Course of Project: Further studies would be concerned with the more complex mapping of various areas in brain as to the effect of cyclic 3',5'-AMP. Further studies would also be undertaken as to the sub-cellular distribution of this nucleotide and to the study of drugs capable of increasing this in brain. Studies would also be directed in the understanding of interaction between various cyclic 3',5'-nucleotides, like cyclic 3',5'-GMP, cyclic 3',5'-UMP and cyclic 3',5'-IMP with cyclic 3',5'-AMP.

Honors and Awards: Dr. Bernard B. Brodie received an honorary doctor of medicine degree from Karolinska Institutet, Stockholm.

Publications: Krishna, G., Hynie, S. and Brodie, B. B.: Effects of thyroid hormones on adenyl cyclase in adipose tissue and on free fatty acid mobilization. Proc. Nat. Acad. Sci. 59: 884-889, 1968.

429

Serial No. NHI-159
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Role of Adenosine 3',5'-Monophosphate in the Central Nervous System

Previous Serial Number: None

Principal Investigators: Dr. George L. Pauk
Dr. Bruce R. Ditzion

Other Investigators: None

Cooperating Units: Dr. Ditzion is a research associate in the Pharmacology-Toxicology Training Program, NIGMS

Man Years:

Total:	1.0
Professional:	1.0
Other:	0.0

Project Description:

Objectives: Adenosine 3',5'-monophosphate is thought to have an important role in the mediation of various hormone actions in many different organ systems. It is known that the central nervous system contains large amoungs of adenosine 3',5'-monophosphate and the enzymes which produce and degrade adenosine 3',5'-monophosphate (adenyl cyclase and adenosine 3',5'-monophosphate phosphodiesterase). However, no function has been determined for this compound in the central nervous system. The objective of this study is to determine if any metabolic relationships of adenosine 3',5'-monophosphate can be determined by direct measurement of this compound in various in vivo and in vitro brain preparations.

Methods Employed: Measurement of adenosine 3',5'-monophosphate is done by a double isotope derivative technique. After addition of C^{14}-labelled adenosine 3',5'-monophosphate, tissue samples are homogenized in cold trichloroacetic acid. The homogenates are then filtered and lipids are extracted with ether. Adenosine 3',5'-monophosphate is then purified by a Dowex 1-X8 formate column, and a paper chromatography with an isopropanol: ammonia:water (6:1:2) solvent system. The adenosine 3',5'-monophosphate is acetylated with H^3-acetic anhydride in the presence of imidazole. Breakdown products and excess tritiated compounds are then eliminated by Dowex 1-X8 formate column chromatography and a final isopropanol:acetic acid: water (7:1:2) paper chromatography. The adenosine 3',5'-monophosphate concentration

is then calculated from ratios of the H^3 to C^{14} in comparison with standards.

It is usually accepted that the best estimates of brain levels of various substrates in vivo are obtained by freezing specimens as rapidly as possible. In this study we have used several methods of obtaining brain tissue including rapid freezing by direct immersion in liquid nitrogen, followed by removal of brain tissue with a chisel.

Major Findings: Studies have been done with male Sprague-Dawley rats and male N.I.H., G.P. mice with similar results. Adenosine 3',5'-monophosphate levels in whole brain of the frozen animals are 1-2nmoles/g of brain. Preparation by decapitation and dissection without freezing yields values 2-3 times basal level. Decapitation with the head dropped directly into liquid nitrogen also yields high values (2-3 times basal level). Pentobarbital anesthesia followed by direct sacrifice in liquid nitrogen gave basal values. Pentobarbital anesthesia does not retard the postmortem rise as measured in the decapitation studies. Animals which were undergoing insulin-hypoglycemic convulsions showed no change in basal levels when sacrificed by immersion in liquid nitrogen. Also, no difference was found in brain adenosine 3',5'-monophosphate levels in fasted animals. The cerebellum was found to have higher levels of adenosine 3',5'-monophosphate than whole brain, which correlates with the finding that phosphodiesterase is low in this area.

Significance to Bio-medical Research and the Program of the Institute: One possible correlation of adenosine 3',5'-monophosphate in brain metabolism may be indicated by the observed rapid fluctuation of adenosine 3',5'-monophosphate levels corresponding with the known changes in glycogenolysis and glycolysis. This finding would support the hypothesis that adenosine 3',5'-monophosphate is involved in activation of brain phosphorylase b kinase. However, since it is also known that adenosine 3',5'-monophosphate is involved in the activation of other enzyme systems in different tissues, it is possible that several enzyme systems might be so related in the brain. The importance of this study to biomedical research is therefore indicated by the possible multiple involvement of adenosine 3',5'-monophosphate as a constant factor in brain metabolism and the potential accessability of this system to pharmacological manipulation.

Proposed Course of Project: The relationship of adenosine 3',5'-monophosphate to neural hormones such as catecholamines in the central nervous system will be studied. Particular emphasis will be given to the relationship to drugs, such as theophylline, dopamine, reserpine, amphetamine and other centrally acting agents. Levels of adenosine 3',5'-monophosphate will be determined after systemic and intracerebral administration of drugs to animals and incubation of brain slices in vitro.

Honors and Awards: None

Publications: None

431

Serial No. NHI- 160
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Role of Adenosine 3',5'-Monophosphate in Arterial Tissue

Previous Serial Number: None

Principal Investigators: Dr. George L. Pauk
Dr. Harriet M. Maling

Other Investigators: Mrs. Martha A. Williams

Cooperating Units: None

Man Years:

Total:	0.05
Professional:	0.04
Other:	0.01

Project Description:

Objectives: Adenosine 3',5'-monophosphate is produced by the adenyl cyclase system which appears to be closely related to the adrenergic "receptors" in many tissues. Some evidence indicates that adenosine 3',5'-monophosphate levels are increased by beta adrenergic type of stimuli and decreased by alpha adrenergic stimuli. Study of the control of adenosine 3',5'-monophosphate levels in arterial tissue should establish whether or not this relationship exists in arterial tissue.

Methods Employed: Adenosine 3',5'-monophosphate was measured by a double isotope derivative assay technique previously described. Arteries obtained under the conditions described below were frozen by immersion in liquid nitrogen, pulverized in the frozen state, and then homogenized in trichloroacetic acid.

Major Findings: Adenosine 3',5'-monophosphate has been measured in a variety of arteries of pentobarbital anesthetized dogs in basal states and during epinephrine infusions. Variable concentrations have been found in the limited data secured presently. A variation of adenosine 3',5'-monophosphate concentration does appear to exist among various arteries with an approximate range of 0.4 to 8.0 mμmoles/g. The highest value has been found in the gracilis artery, which has a pure skeletal muscle vascular bed. Further measurements will need to be correlated with arterial size, the nature of the receptors, and the type of vascular bed.

Epinephrine infusion, producing a blood pressure elevation, was associated with a very slight decrease in adenosine 3',5'-monophosphate content in the carotid, mesenteric, and femoral arteries, when compared to paired artery during control period. If consistent, this may correlate with findings by other investigators studying smooth muscle in other organs.

Significance to Bio-medical Research and the Program of the Institute: There would appear to be important and potentially useful information if this approach would contribute to the understanding of the regulation of arterial tone and therefore blood pressure.

Proposed Course of Project: The preliminary results must be confirmed and other drugs, such as norepinephrine, isoproterenol, and the relationship of alpha and beta adrenergic blocker compounds will be studied. Because of problems of recording the local vascular response *in vivo*, it is planned to perfuse isolated arteries either *in situ* or *in vitro*.

Honors and Awards: None

Publications: None

Serial No. NHI- 161
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Assay of Cyclic 3',5'-AMP and other Cyclic 3',5'-Nucleotides by Gas Liquid Chromatography

Previous Serial Number: None

Principal Investigator: Dr. Gopal Krishna

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.3
Professional:	0.3
Other:	0.0

Project Description:

Objectives: Cyclic 3',5'-AMP has been implicated as a mediator of many important hormone functions. The methods available for its assay so far have been either enzymatic or isotope dilution technique methods, which are very elaborate and time consuming. With the introduction of specific detectors for detecting and estimating of phosphorus compounds in picogram range, the usefulness of GLC in separating and estimating cyclic 3',5'-nucleotides has been investigated.

Methods Employed: Standard GLC techniques were employed.

Nucleotides were quantitatively converted to their respective trimethylsilyl derivative by simple treatment with a silylating agent--bistrimethylsilyl acetamide in acetone at room temperature for 1-2 hours. An aliquot of this was injected directly into GLC.

The column packing [3% OV-17 on GasChrom Q (60-80 mesh)] was chosen after examination of various available packings for their stability at high temperature and for their capacity to resolve complex mixtures of nucleotides. The columns were generally maintained at 240-275°C. An argon flow was 100 ml/min when the argon ionization detector was used. When the phosphorus detector was used the flow rate was 25 ml/min due to certain restrictions of this type of detector. The phosphorus detector consisted of a hydrogen flame detector with a cesium bromide pellet placed into the flame. This detector causes an increased ionization current only when compounds containing phosphorus are

434

burnt in flame while comparatively insensitive to others. This detector is capable of detecting 1 picogram of phosphorus containing compounds with a 10,000-fold dynamic range.

Major Findings: When trimethylsilyl derivative of cyclic AMP was chromatographed on a 4-ft (0.4 cm i.d.) 3% OV-17 on GasChrom Q (60-80 mesh) maintained at 241°C (argon flow 100 ml/min) and measured by the argon ionization detector a single symmetrical peak with a retention time of 8.8 minutes was obtained. Similar type of peaks with different retention times were obtained when 12 other nucleotides were chromatographed under similar conditions. The retention times of these 12 nucleotides are given below.

RETENTION TIME OF TRIMETHYLSILYL DERIVATIVES OF NUCLEOTIDES

Nucleotides	Retention Time (minutes)
2' CMP	1.5
2'(3') UMP	2.4
CYCLIC 2',3' UMP	3.3
2' AMP	4.4
3' AMP	4.4
CYCLIC 2',3' AMP	4.4
CYCLIC 3',5' UMP	4.7
5' AMP	6.0
CYCLIC 2',3' GMP	6.3
CYCLIC 3',5' IMP	7.0
CYCLIC 3',5' AMP	8.8
CYCLIC 3',5' GMP	12.3

It can be clearly seen that cyclic 3',5'-AMP could be separated from other cyclic 3',5'-nucleotides as well as mononucleotides.

The cyclic AMP peak obtained in GLC has been further examined in a mass spectrometer attached to a GLC. Parent ion with a mass unit of 545 corresponding to 3-trimethylsilyl groups attached to cyclic 3',5'-AMP was obtained.

For quantitation of cyclic AMP and other nucleotides, various amounts of TMS derivative were injected into GLC equipped with the argon ionization detector and the peak height of the corresponding peaks was measured. A linear relationship between the quantities injected and peak height was obtained for cyclic 3',5'-AMP ranging from 0.15-7.5 nmoles. Similar results were obtained with 2'-AMP, 5'-AMP, 2'-UMP and cyclic 3 ,5'-IMP.

When a phosphorus detector was used for the assay, the sensitivity of detection of cyclic 3',5'-AMP could be improved. As low as 60 picomole could be detected. Again linear relationships were obtained.

Significance to Bio-medical Research and the Program of the Institute: Cyclic 3',5'-AMP has been known to mediate many of the important functions of various hormones. One of the main criteria for assigning cyclic 3',5'-AMP a

role in any specific hormone action is that the level should change due to the action of hormone on the cell and the change in level should correspond to the effect of hormone. So far there has been great difficulty in separating and extimating cyclic 3',5'-AMP in tissue mainly because of its presence in sub-nanomole range in tissue while other nucleotides are present in micromole range. With the method developed in this laboratory for separation of all other nucleotides by simple precipitation with $Ba(OH)_2$ and $ZnSO_4$ (when cyclic 3',5'-AMP is not removed but obtained quantitatively in the supernatant) in combination with the method of separation and estimation by GLC, it should now be possible to study the hormone function mediated by cyclic 3',5'-AMP. Moreover, it is now possible to use this method to study certain hormone functions which are also mediated by other cyclic 3',5'-nucleotides, especially cyclic 3',5'-GMP, which has been shown to change due to some hormones of pituitary.

Proposed Course of Project: A detailed mapping of cyclic 3',5'-AMP levels in tissues like fat cells, liver, muscle and others under the influence of catecholamines and other hormones would be studied both in control and adrenalectomized animals, where the sensitivity to catecholamines is enormously reduced.

This method would also be employed to study the change of other cyclic 3',5'-nucleotides levels, especially cyclic 3',5 -GMP under the influence of various hormones.

Honors and Awards: None

Publications: None

4 sh

Serial No. NHI- 162
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effect of Peripheral Sympathetic Blockade on Pentylenetetrazol Convulsions in Mice

Previous Serial Number: None

Principal Investigators: Dr. Theodore Koppanyi
Dr. James N. Davis

Other Investigators: None

Cooperating Units: Dr. Koppanyi is on Sabbatical from the Department of Pharmacology, Georgetown University Medical School

Dr. Davis is a Research Associate in the Pharmacology Training Program, NIGMS

Man Years:

Total:	1.6
Professional:	1.6
Other	0

Project Description:

Objectives: To investigate the effect of peripheral sympathetic blockade on the activity of animals as measured by its effect on pentylenetetrazol convulsions.

Methods Employed: Male NIH mice were put in groups of eight each and treated with pentylenetetrazol in combination with sympathetic blocking agents. The animals were observed four at a time for 15 minutes after receiving pentylenetetrazol. Incidence of convulsion was determined as the percent of animals who lost balance associated with a clonic convulsion. FFA was determined by the method of Novak and glucose by the gluco-star test (Worthington Biochemical Corporation).

Major Findings: Pretreatment with the ganglionic blocking agent chlorisondamine was the most potent method of protection against convulsions. Protection occurred with doses as low as 0.1 mg/kg and maximal protection with 3 mg/kg. The norepinephrine release blocking agents, bretylium and BW 392C60, and a β-blocker propranolol, protected against convulsions from 15 minutes to two hours after injection.

437

The anticonvulsant effect of chlorisondamine is reversed by hypodermic injection of either epinephrine or isoproterenol. Plasma levels of free fatty acids and glucose correlate well with the ability of the animal to convulse.

The specific EEG (spike and dome) pattern of pentylenetetrazol was not altered by pretreatment with chlorisondamine. The fact that pentylenetetrazol convulsions can be blocked by interference with the peripheral sympathetic nervous system demonstrated the importance of this system in the ability of the organism to respond to central stimuli.

Significance to Bio-medical Research and the Program of the Institute: These findings correlate well with previous work from this laboratory showing the importance of the peripheral sympathetic nervous system in response to cold and exercise stress. The results may lead to a more complete understanding of the role of this system in normal activity.

Proposed Course of Project: Further studies will be undertaken to examine the role of the central sympathetic nervous system in pentylenetetrazol convulsion.

Honors and Awards: None

Publications: None

Serial No. NHI-163
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Nature of the Central Actions of Caffeine and Theophylline

Previous Serial Number: NHI-363 (1967); NHI-357 (1966)

Principal Investigators: Dr. Harriet M. Maling
Dr. James R. Gillette

Other Investigators: Mr. Amnuay Thithapandha
Mrs. Martha A. Williams
Mr. Wilford Saul

Cooperating Units: Mr. Thithapandha is a graduate student at George Washington University, supported by the Rockefeller Foundation.

Man Years:

Total:	1.0
Professional:	0.2
Other:	0.8

Project Description:

Objectives: Interactions of caffeine and theophylline with other drugs have been studied to gain insight into the nature of the central actions of the xanthines. Measurements of confinement motor activity after injections of caffeine have been correlated with brain levels of caffeine. Experiments have been designed to test the hypothesis that caffeine and theophylline act like a central sympathomimetic drug such as d-amphetamine.

Methods Employed: The mild stimulation of the central nervous system produced by caffeine and theophylline has been measured in terms of confinement motor activity (CMA). In most of the experiments completed in 1967-1968, CMA was measured visually, with the observer counting the number of "up and down" movements made by a rat when confined to a cage too small to permit extensive horizontal movement. At present, CMA is measured automatically in an apparatus modified from that described by Tedeschi et al (J. Pharm. Sci. 53:1046, 1964). Our apparatus for measuring CMA of 6 rats individually and simultaneously was designed and constructed by Mr. Glenn Rahmoeller of the Biomedical Engineering Instrumentation Branch.

Major Findings: Caffeine, theophylline, d-amphetamine, and other centrally acting sympathomimetic drugs increased the CMA of rats. This increase in CMA seemed to parallel the degree of alertness. CMA was not increased by the antidepressant DMI, the convulsants picrotoxin and pentylenetetrazol, and the hallucinogenic agents, LSD and mescaline. CMA scores after caffeine were reduced by pretreatment with the alpha adrenergic blocking drugs, phentolamine, phenoxybenzamine, and chlorpromazine, and by the beta adrenergic blocking drug, propranolol. Blockade of norepinephrine synthesis by α-MT reduced the effect of caffeine. Depletion of brain amines by repeated doses of reserpine completely blocked the central effects of caffeine. Cocaine potentiated the CMA produced by caffeine or d-amphetamine. Caffeine and d-amphetamine produced additive effects. These interactions with other drugs support the hypothesis that caffeine and theophylline act like central sympathomimetics such as d-amphetamine.

The brain levels of caffeine or theophylline at the time of peak effect were 10-15 μg/g of brain. Higher brain levels were associated with toxicity, as indicated by lower CMA scores. After the peak effect had been reached with non-toxic doses, there was a good correlation between brain levels of caffeine and CMA score.

Significance to Bio-medical Research and the Program of the Institute: Interactions of caffeine and theophylline with other drugs may occur frequently in humans, because xanthine beverages, especially coffee and tea, are widely used. The possibility of unplanned interactions in man creates interest in experimental studies of interactions in rats.

Proposed Course of Project: Additional experiments are planned to determine more completely dose-response relationships and the duration of effects. Brain levels of caffeine will be measured at various times after intraperitoneal administration.

Honors and Awards: None

Publications: None

Serial No. NHI-164
1. Chemical Pharmacology
2. Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Role of the Sympathetic Nervous System and the Adrenal Cortex in Inflammation

Previous Serial Number: None

Principal Investigators: Dr. Harriet M. Maling
Dr. Marion E. Webster

Other Investigators: Mrs. Martha A. Williams
Mr. William Anderson, Jr.

Cooperating Units: Dr. Webster and Mr. Anderson are members of the Laboratory of Clinical Biochemistry, National Heart Institute

Man Years:

Total:	0.2
Professional:	0.1
Other:	0.1

Project Description:

Objectives: It is well-known that sympathectomized and adrenalectomized animals are more sensitive to a variety of agents and conditions leading to systemic circulatory shock. Since systemic shock involves widespread collapse of the microcirculation, adrenalectomized and sympathectomized animals may also be hypersensitive to conditions and agents causing localized disturbances of the microcirculation, as in inflammation. This project is a study of the effects of chemical sympathectomy and adrenalectomy on inflammation of the paw induced by subcutaneous injection of cellulose sulfate or carrageenin.

In a related project in collaboration with Dr. Webster, we are studying the role of kinins in inflammation of the rat's paw produced by the injection of uric acid crystals. This project is described in the Annual Reports of the Laboratory of Clinical Biochemistry.

Methods Employed: Cellulose sulfate or carrageenin was injected into the right hind paw. Inflammation was measured by amputating both hind paws and weighing them. The difference in weight of the hind paws was a measure of the inflammation produced by the injected substance.

Major Findings: Adrenalectomized (ADX) rats were more sensitive to carrageenin, but not to cellulose sulfate, than normal rats. The increased response to carrageenin was more striking in ADX rats maintained on saline than in ADX rats maintained on distilled water for 3 days before use. There was no clear difference in response to cellulose sulfate and to carrageenin between normal and adrenal demedullated rats. But blockade of the sympathetic nervous system in demedullated rats reduced the inflammatory response to carrageenin. The reduction in inflammatory response was more striking when blockade of the sympathetic nervous system was achieved with the ganglionic blocking agent, chlorisondamine, than when release of norepinephrine at adrenergic nerve endings was prevented with the bretylium-like drug, BW392C60. Blockade of the sympathetic nervous system in intact rats did not reduce the inflammatory response as much as blockade in demedullated rats.

Significance to Bio-medical Research and the Program of the Institute: These studies increase our understanding of the effects of chemical sympathectomy and adrenalectomy. They may also enable us to understand the effects of autonomic drugs in inflammation.

Proposed Course of Project: We shall compare in intact and adrenal demedullated rats the effects on inflammation produced from blockade of the sympathetic nervous system by depletion of norepinephrine content of peripheral tissues. Reserpine and syrosingopine will be used to deplete the norepinephrine content.

Honors and Awards: None

Publications: None

Serial No. NHI- 165
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Pharmacological Actions of ST-155 (Catapres^{R}) and Other Imidazolines in the Rat

Previous Serial Number: NHI-337

Principal Investigators: Dr. Harriet M. Maling
Dr. Arthur Cho

Other Investigators: Mrs. Martha A. Williams

Cooperating Units: None

Man Years:

Total:	0.6
Professional:	0.2
Other:	0.4

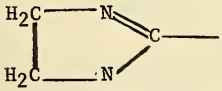
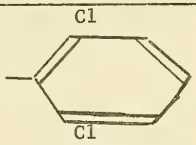
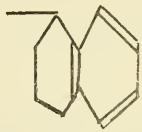
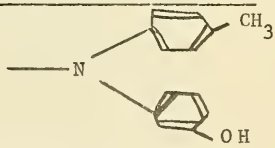
Project Description:

Objectives: ST-155 (2-(2,6-dichlorphenylamine)-2-imidazoline) is a potent imidazoline now under clinical trial in this country as an antihypertensive drug. Doses as low as 1 μg/kg orally are effective in man. The most prominent side effect is sedation. We have previously suggested that ST-155 may block adrenergic activity centrally, thus resembling chlorpromazine, even though it acts like a sympathomimetic drug peripherally. The peripheral sympathomimetic effects are not surprising because ST-155 is closely related to several imidazoline sympathomimetic drugs which are sold as nasal decongestants. In this project, several pharmacological actions of ST-155 are compared with those of two imidazoline sympathomimetics (naphazoline and tetrahydrozoline) and two imidazoline alpha adrenergic blocking drugs (tolazoline and phentolamine). The structures of ST-155 and the other imidazolines used in this study are given in Table I.

We have compared the effects of ST-155 and these related imidazolines on the cardiovascular responses to tilt, which is usually assumed to involve the same pathways as the carotid occlusion reflex. We chose to compare effects on a reflex response since the hypotensive action of ST-155 is most probably centrally mediated.

The sedative action of ST-155 is difficult to measure in rats. We have looked for a sedative effect and a central adrenergic blocking action by measuring the effect of treatment with ST-155 on the increased confinement motor activity induced by d-amphetamine.

TABLE I. STRUCTURES OF ST-155 AND RELATED IMIDAZOLINES

	—A—	—B—
ST-155	—NH—	
Naphazoline	—CH_2—	
Tetrahydrozoline	—	
Tolazoline	—CH_2—	
Phentolamine	—CH_2—	

Methods Employed: Each imidazoline was tested for its effect on arterial blood pressure during "tilt" in rats anesthetized by the intraperitoneal injection of a combination of chloralose, 60 mg/kg, and urethane, 600 mg/kg. Preliminary experiments showed that the tilt reflex was more stable in rats anesthetized with chloralose-urethane than in rats anesthetized with a barbiturate. Arterial blood pressure was recorded on a Grass polygraph with a Statham P23D transducer, connected to a polyethylene catheter inserted into a femoral artery. The rat was strapped to a wooden board. The tilt reflex was tested by raising the board from a horizontal position

to a vertical position. The rat was kept in this vertical position with head up and feet down for 1 minute. The tilt reflex was tested at suitable intervals during the experiment, usually once every 15 minutes.

The imidazolines were tested for blockade of central adrenergic activity by measuring their effectiveness in antagonizing the increased confinement motor activity (CMA) produced by d-amphetamine sulfate (2 mg/kg, i.p.). CMA was defined by Tedeschi et al. (J. Pharm. Sci. 53:1046, 1964) as the "up and down" movements made by a rat when confined under standard conditions in a cage too small to permit extensive movement in a horizontal plane. In our earlier experiments we measured CMA by visual observation. Now we measure CMA of 6 rats simultaneously in an apparatus designed and constructed by Mr. Glen Rahmoeller (Biomedical Engineering Instrumentation Branch). This apparatus is modified from the design described by Tedeschi et al.

Major Findings: ST-155 in small doses (10-30 µg/kg i.p.) inhibited the tilt reflex, as shown by a fall in blood pressure during tilt. In contrast, small doses (1-30 µg/kg, i.p.) of naphazoline, tetrahydrozoline, and tolazoline either did not affect the tilt response or increased the pressor response to tilt. Small doses of phentolamine produced variable effects, with a depressor response to tilt in 2 rats and a pressor response in 3 rats. Large doses of ST-155 (300 µg/kg i.p.) produced an early rise in arterial pressure and an augmented pressor response to tilt. After the initial rise in arterial pressure subsided, the pressure fell below pre-injection levels and the response to tilt became depressor.

Since ST-155 causes sedation in man, it seemed reasonable to test whether ST-155 could reduce the increased CMA induced by d-amphetamine (2 mg/kg i.p.). This hypothesis was tested in a 6 x 6 Latin square, with 6 treatments compared in each block of the experiment and all 6 treatments compared in each cage of the CMA apparatus. This experimental design eliminates errors due to variation among different groups of rats and from cage to cage in the apparatus. Analysis of our Latin square data showed that 20 µg/kg of ST-155 did not significantly modify the CMA induced by d-amphetamine, but doses of 100 and 500 µg/kg appreciably decreased the activity. Another Latin square experiment was then set up to compare the effects of all 5 imidazolines, each in a dose of 300 µg/kg. There was a slight reduction of d-amphetamine-induced activity with tolazoline and ST-155, but no obvious effect with naphazoline, tetrahydrozoline, and phentolamine. This experiment should be repeated with higher doses. The sedation produced by ST-155 in rats is peculiar in that the rats are excessively irritable and show a greater tendency than usual to bite when handled.

Both small (30 µg/kg) and large (300 µg/kg) doses of ST-155 elevate plasma glucose markedly. Peak glucose levels were 290 mg% after the larger dose and 255 mg% after the small dose. Hyperglycemia was also produced by ST-155 in the hypophysectomized rat, but peak levels were lower than in the intact rat.

Hypothermia was produced consistently by large doses of ST-155 (1 mg/kg i.p.). Hypothermia has also been reported by others for naphazoline and tetrahydrozoline.

We conclude that large doses of ST-155 in rats produce all the pharmacological effects which have been reported for other imidazolines. These effects include alpha adrenergic stimulation, hyperglycemia, sedation, bradycardia and hypothermia. The sedation and bradycardia suggest a central action. The sedation is most clearly demonstrated by a partial blockade of the increased CMA produced by d-amphetamine. ST-155 differs from the other imidazolines in that small doses consistently produce a depressor response to tilt.

Significance to Bio-medical Research and the Program of the Institute: ST-155 is now under clinical trial in this country for the treatment of hypertension. It is the most potent antihypertensive drug known. This comparison of its pharmacological actions with those of other imidazoline drugs permits a better evaluation of its properties.

Proposed Course of Project: The comparison of the actions of ST-155 with those of other imidazolines will be completed.

Honors and Awards: None

Publications: None

Serial No. NHI- 166
1. Chemical Pharmacology
2. Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies in Mice of Barbituric Acid, Thiobarbituric Acid, 5-(1,3-Dimethylbutyl)-5-Ethyl Barbituric Acid, and 5,5-Diphenyl Barbituric Acid

Previous Serial Number: None

Principal Investigators: Dr. Theodore Koppanyi
Dr. Harriet Maling

Other Investigators: Mrs. Martha A. Williams

Cooperating Units: Dr. Koppanyi is a guest worker in LCP, on sabbatical leave from his position as Professor of Pharmacology at Georgetown University Medical School

Man Years:

Total:	0.1
Professional:	0.05
Other:	0.05

Project Description:

Objectives: In this study, we seek experimental data to support the statement that barbituric acid and thiobarbituric acid are inert. In addition, we wish to study the properties of two barbiturates which have not been adequately studied. These barbiturates are 5-(1,3-dimethylbutyl)-5-ethyl barbituric acid (DMBEB) and 5,5-diphenyl barbituric acid (DPB). Interactions of these barbiturates with pentobarbital will be studied, to test the prediction that treatment with a weak barbiturate may protect an animal from a potent barbiturate by preventing access to receptors which have been blocked by the weak barbiturate.

Methods Employed: We observe the effects of i.v. and i.p. injection of these drugs upon the behavior of mice.

Major Findings: Anesthesia is not produced by doses of thiobarbituric acid as great as 500 mg/kg i.p. Such doses cause death within 24 hours, but the cause of death is not clear. Death may be caused by the injection of this insoluble compound in an acid solution. The cause of death requires further study. Anesthesia is not produced by doses of barbituric acid as great as 1.0 g/kg. Large doses of DPB (400 mg/kg i.v.) produce anesthesia, with a slow onset of effect and prolonged duration. Smaller doses of DPB

447

(100-200 mg/kg), which in themselves produce no obvious effect, prolong markedly the duration of anesthesia produced by pentobarbital sodium. DMBEB (22.5 mg/kg i.p.) produces convulsions. Despite its convulsant activity, DMBEB increases the depth and prolongs the duration of anesthesia produced by pentobarbital sodium.

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

Experimental studies of interactions of weak barbiturates and potent barbiturates may lead to new methods of treating barbiturate poisoning and treating convulsions. A weak barbiturate with long duration of action may be useful in the treatment of epilepsy.

Proposed Course of Project: Our findings so far have been based on exploratory tests. Our findings must be confirmed with sufficient data for statistical evaluation.

Honors and Awards: None

Publications: None

Serial No. NHI- 167
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of Action of Antihistamines

Previous Serial Number: None

Principal Investigators: Dr. George A. Clay
Dr. Theodore S. Koppanyi

Other Investigators: Dr. Harriet M. Maling
Mr. Wilford Saul

Cooperating Units: Dr. Koppanyi is on Sabbatical leave from Georgetown University.

Man Years:

Total:	0.8
Professional:	0.7
Other:	0.1

Project Description:

Objectives: Antihistamines have been shown to inhibit the active transport mechanism for norepinephrine in the heart and trachea, and to alter the metabolism of histamine by inhibition of methyl histamine transferase in purified enzymatic preparations. The purpose of this study was to examine the in vitro effects of antihistamine on the heart and the trachea in guinea pig, and to see if the accumulation and/or the metabolism of histamine were altered by antihistamines in these tissues.

Methods Employed: Endogenous histamine levels were determined enzymatically. The accumulation and metabolism of histamine in isolated tissues were measured by standard isotopic techniques. The biologic activity of antihistamine was measured in vitro on the guinea pig ileum and the guinea pig trachea ring.

Major Findings: (1) Through the use of isotopes, it was shown that histamine is not actively taken up into the isolated heart and trachea as is norepinephrine. Blockade of energy-dependent transport mechanisms did not alter the level of accumulation of histamine in the tissue, suggesting that histamine may accumulate in these tissues by passive diffusion. Similarly, none of the antihistamines tested diminished the concentration of histamine in tissue.

(2) Antihistamines did not significantly alter the metabolism of histamine in isolated tissues when used at concentrations shown to block the actions of histamine by bioassay. Antihistamine levels known to inhibit histamine methytransferase were well above effective doses.

Significance to Bio-medical Research and the Program of the Institute: These findings may lead to a better understanding of the mechanism of actions of antihistamines.

Proposed Course of Project: The single tracheal ring assay developed will be applied to the study of the actions of sympathomimetic drugs.

Honors and Awards: None

Publications: None

Serial No. NHI- 168
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Blood and Plasma Histamine Levels after Compound 48/80 and the Role of Histamine in Brief Muscle Contraction

Previous Serial Number: NHI-369 (1967) and NHI-347 (1966)

Principal Investigators: Dr. Harriet M. Maling
Dr. Michael A. Beaven

Other Investigators: Mr. Wilford Saul
Mrs. Martha A. Williams

Cooperating Units: None

Man Years:

Total:	1.2
Professional:	0.2
Other:	1.0

Project Description:

Objectives: This project was undertaken to test the hypothesis that a release of histamine from normal skeletal muscle during contraction may contribute to the vasodilatation associated with the contraction. We experienced difficulty, however, in measuring the histamine content of the venous effluent. Considerable time was spent in increasing the sensitivity of the enzymatic method. The experiments with compound 48/80 were undertaken as a test of our ability to measure release of endogenous histamine.

Methods Employed: Blood and plasma levels of histamine were measured enzymatically. Dogs were anesthetized with pentobarbital sodium for experiments with the isolated gracilis muscle and with chloralose-urethane (50 mg/kg chloralose and 500 mg/kg urethane) for the experiments with Compound 48/80.

Major Findings: In conscious dogs, blood levels of histamine averaged 11.5 ± 2.7 ng/ml (8 dogs). Plasma levels in these same dogs were much lower (3 ng/ml or less). Anesthesia caused an initial increase in blood histamine levels, to about twice the level in the conscious animal. After about 30 minutes of pentobarbital anesthesia, the blood content of histamine had returned to the preanesthetic level.

45

In experiments with Compound 48/80 (1 mg/kg i.v.), peak histamine levels in plasma and blood were reached within 1 min., with plasma levels (800 ng/ml) about twice the levels in blood. Plasma and blood histamine levels after Compound 48/80 declined exponentially during the first 15 minutes, but changes during the next 150 minutes were more complex. The initial declines corresponded to half-lives of 5-10 minutes for plasma and 9-20 minutes for blood. The lowest level of arterial blood pressure also occurred within 1 minute, with a slow return to preinjection level in about 90 minutes. The hematocrit rose from about 45 to 65 in 15 minutes and remained at that level for the duration of the experiment. A second injection of 48/80 two hours after the first injection caused a small fall in blood pressure with no measurable change in histamine content of blood and plasma.

In experiments with the isolated gracilis muscle, control histamine levels in the venous effluent were about the same as in the conscious dog. The histamine content of the venous effluent increased 50 to 100% during some periods of nerve stimulation, but increases did not occur invariably. Additional experiments are required to determine the relationship between nerve stimulation and histamine content of the venous effluent.

Enzymatic measurement of the histamine content of gracilis muscles from two dogs gave values of 1.0 and 1.4 $\mu g/g$, somewhat lower than the value of 1.72 ± 0.19 $\mu g/g$ obtained by fluorometric measurements in 5 dogs. There was no difference in histamine content of the perfused muscle and control muscle from the opposite side.

Significance to Bio-medical Research and to the Program of the Institute: This project should increase our understanding of the role of histamine in muscle physiology.

Proposed Course of Project: Additional experiments will be performed with the isolated gracilis muscle to clarify the relationship between histamine content of the venous effluent and vasodilatation accompanying muscle contraction produced by nerve stimulation.

We hope to use the isolated perfused gracilis muscle as a tool in other studies. For example, we hope to collaborate with Dr. Pauk and Dr. Krishna on changes in muscle and arterial content of adenyl cyclase and cyclic AMP produced by intraarterial infusions of norepinephrine, isoproterenol, theophylline, and other drugs.

Honors and Awards: None

Publications: None

Serial No. NHI-169
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigation of the Role of Histamine in the Salivary Gland: Studies on Histamine Formation.

Previous Serial Number: NHI-376

Principal Investigators: Dr. Sten Jacobsen, Dr. Walter B. Severs, and Dr. Michael A. Beaven

Other Investigators: None

Cooperating Units: Dr. Walter B. Severs is a U.S.P.H.S. Postdoctoral Fellow

Man Years:

Total:	1.4
Professional:	1.4
Other:	0

Project Description:

Objectives: Previous reports from this laboratory have demonstrated that nonmast cell stores of histamine can be labeled by systemic administration of ^{3}H-histamine. Data obtained by labeling the cat submaxillary gland with ^{3}H-histamine was consistant with the view that histamine could be a mediator in submaxillary gland function. Apparent histamine turnover was of the order of 2-3 mcg/g tissue and appeared to be directly related to the level of tissue activity; *i.e.*, increased turnover rate during stimulation and decreased turnover rate at rest. The submaxillary gland was not depleted of its histamine stores during physiological activity, thus implying that histamine synthesis was occuring in the gland at a rate proportional to utilization. During the past year, it was the purpose of this project to critically evaluate whether or not the functioning submaxillary gland was capable of synthesizing histamine, a prime requirement before the role of "mediator" could be assigned to this compound.

Methods Employed: A perfusion system for cat submaxillary glands was developed. Briefly, a submaxillary gland from an adult male cat was exposed and circulatory vessels supplying the gland isolated. Blood from the individual animals was pumped at known pressure and volume from a disc oxygenator into the artery. Venous blood from the gland was returned to the oxygenating unit. Blood supplying the arterial circuit was maintained at 37°C, and O_2 tension, CO_2 tension and pH were within physiological limits. If the perfusion was to

453

be prolonged, glucose was added hourly to prevent hypoglycemia. Addition of cholinergic drugs to the blood produced a suitable flow of saliva which was monitored via a drop counter from the cannulated salivary duct.

Side-chain labeled ^{3}H-histidine of high specific activity was mixed with the blood reservoir. Blood samples from the arterial and venous circuits and saliva samples were obtained at various times thereafter. At the termination of experiments, the gland was removed and homogenized in 0.1 M acetate buffer, pH 4.5. In these experimentsthe saliva, glandular homogenate, and various blood samples were then assayed for both labeled and endogenous histidine and histamine. The samples were also assayed for labeled methylhistamine. The assay methods utilized were as follows: 1. Histamine was converted to methylhistamine by the method developed by Snyder *et al.* (J. Pharmacol. exp. Therap. 153: 544, 1966) and modified by Jacobsen and Beaven (1967 Annual Report #NHI-375); 2. ^{3}H-methylhistamine was determined by carrying samples of the biological material through the extraction procedure of the histamine assay; 3. Histidine was determined by incubating samples of biological material with bacterial histidine decarboxylase (histidine and histamine free) and assaying for total histamine. Subtraction of endogenous histamine from total histamine gives the amount of histamine derived from histidine.

It was possible to utilize the perfusion system described to study possible histamine formation using ^{14}COOH-L-histidine as a substrate. This was done by making the system gas-tight and passing the gas from the oxygenator through hyamine hydroxide to trap CO_2. Carbon dioxide was collected initially when the submaxillary gland was at rest, and also when cholinergic stimulation was carried out. This procedure is advantageous as the catabolism of formed histamine would not mask synthesis, as the $^{14}CO_2$ evolved is the measured parameter rather than labeled histamine. As in experiments with side-chain labeled ^{3}H-histidine, blood and glandular histamine and histidine were assayed.

Major Findings: 1. Perfusion of the cat submaxillary gland with whole blood containing ^{3}H-histidine resulted in a labeling of the glandular histidine. Radioactive histamine was not found in the gland although estimation of the specific activity of glandular histidine indicated that labeled histamine would have been detected if the synthesis rate were greater than 0.1 mcg/g/hr. Synthesis was not observed even though the gland appeared viable and was functioning. Similarly, ^{3}H-methylhistamine was not detected in the gland. Venous blood from the gland was also devoid of labeled histamine and methylhistamine. On comparing the histamine levels of the perfused and nonperfused glands from the same cat, no evidence of histamine depletion was obtained. 2. The saliva obtained in these experiments contained ^{3}H which has tentatively been identified as water and uroconic acid. 3. Studies with ^{14}COOH-L-histidine in the perfusion system confirmed that synthesis of histamine by the submaxillary gland did not occur but that whole blood formed histamine at a rate of approximately 50 ng/ml/hr. 4. Studies indicated that urocanic acid significantly interfers with the assay of labeled histamine by the benzene sulfonyl derivative technique. This finding accounts for the apparent histamine formation described in last year's annual report.

454

Significance to Bio-medical Research and the Program of the Institute: The results obtained in this study indicate that the intact functioning submaxillary gland of the cat does not synthesize histamine, although experiments measuring ^{3}H-histamine decline from the tissue suggest a high rate of formation. The nature of the glandular histamine store does not appear to be in a simple steady state wherein utilization of histamine is immediately balanced by an equal synthesis. The observation that blood was synthesizing histamine suggests that it may be involved with the origin of tissue histamine.

Proposed Course of Project: Studies to elucidate the role of histamine in submaxillary gland will be continued.

Honors and Awards: None

Publications: None

Serial No.-NHI-170
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH

Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Formation of Histamine in Tissues: Possible Mechanism Controlling the Rate of Histamine Formation

Previous Serial Number: NHI-374

Principal Investigators: Dr. Sten Jacobsen, Dr. Walter B. Severs, and Dr. Michael A. Beaven

Other Investigators: None

Cooperating Units: Dr. Walter B. Severs is a U.S.P.H.S. Postdoctoral Fellow.

Man Years:

Total:	1.4
Professional:	1.4
Other:	0

Project Description:

Objectives: In several laboratories studies with radioactive histamine have shown that this compound has a rapid turnover rate in many tissues. Furthermore, the turnover rate is influenced by the level of physiological activity of the individual tissues even though it has not been possible to demonstrate histamine synthesis in certain tissues by injection of the labeled precursor, histidine. In tissues where histamine synthesis can be demonstrated, such as rat gastric mucosa, little is known regarding the regulation of the activity of histidine decarboxylase, the enzyme specifically concerned with the synthesis of histamine from histidine. In another report the observation was made that histamine is synthesized in blood. This report presents data suggesting that the uptake of L-histidine in blood cells might be a rate limiting step in the formation of histamine and describes studies assessing the possibility that the uptake of histidine into gastric mucosa is an important factor in the regulation of histamine synthesis.

Methods Employed: (1) *Blood studies.* Whole blood was withdrawn from rats under ether anesthesia into heparinized syringes. After equilibration of the blood with 95% O_2 - 5% CO_2, ^{14}COOH-L-histidine was added and the blood was incubated at 37°C and $^{14}CO_2$ evolved during the incubation was trapped in hyamine hydroxide. The amount of $^{14}CO_2$ evolved was an index of histamine formation. The effects of several metabolic inhibitors were studied using this system. In other experiments, leucocytes were isolated and then resuspended in cell-free plasma or an isotonic buffer medium. Uptake of ^{3}H-L-histidine by the erythrocytes and leucocytes of blood was also investigated by assaying the ^{3}H-L-histidine by methods described in other reports.

(2) *Rat stomach studies.* Stomachs from fasted and fed animals were excised, the muscular tissue cut into slices of 0.5 mm thickness and further divided as required. Tissue incubations were carried out in Krebs Ringer bicarbonate solution in the presence of ^{14}COOH-L-histidine to determine histamine formation. The effects of several transport inhibitors on histamine formation were studied. In other experiments, uptake of side-chain ^{3}H-L-histidine was investigated. In addition, the effects of methionine on histamine formation by rat stomach homogenate and partially purified rat stomach histidine decarboxylase was determined.

Major Findings: (1) Rat blood is capable of synthesizing histamine at rates varying from approximately 50-150 ng/ml/hr. Leucocytes appear to be the major cellular component in which synthesis occurs, and these cells accumulate ^{3}H-histidine from plasma much more rapidly than do erythrocytes. We have estimated that as much as 85% of leucocyte histidine may turn over in one hr. In studies using transport inhibitors, it was found that at concentrations of 1 mM, ouabain, methionine, and iodoacetic acid inhibit histamine formation in whole blood by about 40%. At 10 mM,methionine inhibited synthesis by more than 80%. These findings raised the possibility that substrate transport is the rate-limiting step in the synthesis of histamine by leucocytes.

(2) The amount of histamine formed by rat stomach slices was several times greater in slices from fed rats than in those from starved rats. Uptake of ^{3}H-histidine and sensitivity to methionine inhibition also appeared to be related to the state of feeding. Thus when the methionine content of the medium was ten times greater than that of histidine, the rate of both histamine synthesis and uptake of ^{3}H-histidine by slices from fed animals was decreased to values near those observed in tissue from fasted rats. Methionine did not appreciably decrease the rate of histidine uptake or histamine synthesis by slices from fasted rats. Since methionine did not affect the activity of the purified enzyme to the same extent, it seems likely that methionine affects histamine synthesis by reducing the uptake of histidine. These data suggest that substrate transport may be a rate-limiting step in histamine synthesis. Cysteine appeared to produce similar effects to methionine. Histamine synthesis by stomach slices did not appear to be influenced by sodium-free medium or iodoacetic acid (10 mM).

Significance to Bio-medical Research and the Program of the Institute: These studies have demonstrated that blood components (especially leucocytes) are a possible source for histamine in body tissues. Using both rat blood and rat stomach as test systems, we have demonstrated that regulation of histidine entry into cells could be a mechanism controlling histamine synthesis The effects of methionine, reported herein, provides a useful tool in exploring these mechanisms.

Proposed Course of Project: Studies will continue to determine the factors regulating entry of histidine into cells and their relationship to histamine synthesis.

Honors and Awards: None

Publications: Beaver, M.A., Horakova, Z., Severs, W.B., and Brodie, B.B.: Selective labeling of histamine in rat gastric mucosa: application to measurement of turnover rate. J. Pharmacol. Exp. Therap., in press, 1968.

Serial No. -NHI-171
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Interaction of Gastrin and Histamine in Gastric Secretion

Previous Serial Number: None

Principal Investigators: Dr. J. Leme

Other Investigators: Dr. M. A. Beaven

Cooperating Units: During the first $4^1/_2$ months Dr. Leme was supported by a Guggenheim Fellowship.

Man Years:

Total:	0.6
Professional:	0.6
Other:	0

Project Description:

Objectives: To investigate the extent to which the stimulation of gastric secretion by gastrin is correlated to mobilization of gastric histamine.

Methods Employed: Tritium-labeled histamine was injected intravenously into rats and the animals killed at various times thereafter. Stomachs were removed and labeled and endogenous histamine determinations performed. The benzenesulfonyl derivative technique (Schayer *et al.*, 1958) was used to estimate labeled histamine and the fluorimetric procedure (Shore *et al.*, 1959) for endogenous histamine. Decarboxylase activity of stomach homogenates was determined according to the method described by Levine and Watts (1963) by trapping in hyamine the $^{14}CO_2$ evolved during incubation with carboxyl-labeled ^{14}C-L-histidine. To determine the acid content of gastric juice the pyloric and esophagic openings of the stomach were ligated and the animals killed at various times. The ligated stomachs were removed and samples of juice titrated with NaOH using bromphenol blue as an indicator.

Major Findings: Gastrin (5 µg, s.c.) administration was followed by an increase in the rate of disappearance of labeled histamine suggesting that histamine was mobilized at a greater rate. At the same time the levels of endogenous histamine were reduced. The reduction in histamine levels was followed by an increase in decarboxylase activity in stomach leading to a replenishment of the endogenous stores of histamine. There was no further depletion of histamine levels when another dose of gastrin was given. However, the second dose of gastrin always evoked increased gastric acid output. This result suggests that the decarboxylase activity induced by the first dose of gastrin was sufficient to maintain the histamine levels upon further mobilization of histamine.

Significance to Bio-medical Research and the Program of the Institute: This project can contribute to a better understanding of the mechanism of action of gastrin as a stimulant of gastric secretion.

Proposed Course of Project: This project will be terminated.

Honors and Awards: None

Publications: None

Serial No. - NHI-172
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Analysis of the Mechanism of Gastric Histamine Depletion by Reserpine

Previous Serial Number: None

Principal Investigators: Dr. Lawrence Isaac

Other Investigators: Dr. M. A. Beaven, Dr. A. K. Cho, and Mrs. B. Eaton Hodshon

Cooperating Units: Dr. Isaac is a Research Associate in the Pharmacology Toxicology Program, NIGMS.

Man Years:

Total:	0.2
Professional:	0.2
Other:	0

Project Description:

Objectives: Reserpine is known to decrease histamine levels in gastric mucosa as well as catecholamines in the adrenergic system. The finding that histamine is present in the non-particulate supernatant of stomach homogenates, whereas catecholamines are localized in subcellular vesicles suggests the possibility that reserpine acts differently on these amine systems. The present study was undertaken to determine the mechanism of action of reserpine on the decrease of gastric histamine.

Methods: Histamine was measured after its enzymatic conversion to methylhistamine using C^{14}-S-adenosylmethionine and guinea pig brain methylating enzyme. The C^{14}-methylhistamine formed was extracted into $CHCl_3$ and counted in a liquid scintillation spectrometer. Histamine is expressed as µg/g of glandular stomach. Gastric histidine decarboxylase was measured in stomach homogenates by collecting $C^{14}O_2$ produced by decarboxylation of $C^{14}OOH$-histidine added to the homogenate. Decarboxylase activity is expressed as nanomoles $C^{14}O_2$ formed/100 mg tissue/90 min incubation. In some studies, bilateral gastric vagotomy was used to determine the effects of reserpine in the absence of parasympathetic tone.

Major Findings: After administration of reserpine to rats feeding ad lib, histamine levels decline from 32 µg/g to 20 µg/g between 2 to 5 hrs post injection. In addition, histidine decarboxylase activity falls from

461

about 5.4 nmole to about 1.3 nmoles. This effect is noticeable 15 min after injection of reserpine and reaches its peak decline in 1 hr. Thus, the decrease in decarboxylase activity precedes the decline in histamine levels. Refeeding of fasted rats causes decarboxylase activity to increase but reserpine blocks this activation. After refeeding of fasted rats for 3 hr, the decarboxylase activity returns to the normal range, and reserpine now causes a decrease of about 60% within 1 hr. Histamine levels decline similarly in control and treated animals indicating the refeeding episode causes histamine mobilization to overshadow the reserpine effects.

Bilateral gastric vagotomy had no effect on the 60% reduction in decarboxylase activity after reserpine.

Significance to Bio-medical Research and the Program of the Institute: Reserpine may prove a valuable tool in studying the relationship between histamine synthesis and physiological functions. In addition, the findings described above emphasize that reserpine evokes actions other than depletion of catecholamines and serotonin.

Proposed Course of Project: Further studies will be undertaken to find the mechanism of reserpine action. Effects of reserpine on the V_{max} and K_m of histidine decarboxylase will be determined. The action of reserpine in animals pretreated with protein synthesis inhibitors such as cycloheximide will be studied particularly in relationship to its effect on histamine levels and gastric secretion.

Honors and Awards: None

Publications: None

462

Serial No. NHI-173
1. Chemical Pharmacolog
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Enzymatic Mechanisms concerned with the Metabolism of Histamine

Previous Serial Number: None

Principal Investigators: Dr. Michael A. Beaven, Dr. Walter B. Severs, and Dr. Sten Jacobsen

Other Investigators: None

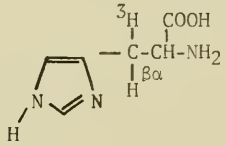
Cooperating Units: Dr. Severs is a U.S.P.H. Postdoctoral Fellow.

Man Years:

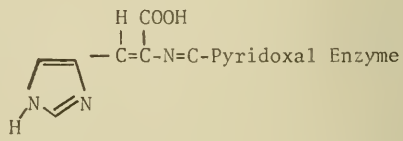
Total:	0.4
Professional:	0.4
Other:	0

Project Description:

Objectives: To study the mechanisms by which histamine is formed from L-histidine and histamine is deaminated to form imidazole acetic acid. A number of mechanisms have been proposed for the decarboxylation of histidine by histidine decarboxylase and deamination of histamine by histaminase, but there is no experimental evidence to support any of these proposed mechanisms. The recent availability of L-histidine labeled in the side chain with ^{3}H [I] made it possible to determine whether the H on the β-carbon is labelized during decarboxylation of the amino acid indicating that the intermediate in this reaction is the unsaturated compound [II] as proposed by Werle and Pechmann (1949).



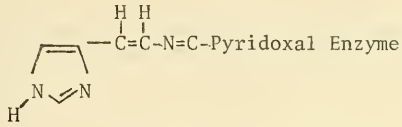
[I]



[II]

463

Similarly, by preparing side chain labeled histamine from the labeled histidine using bacterial decarboxylase which is not a pyridoxal enzyme, it is possible to determine whether the intermediate compound in the deamination of histamine by histaminase is the following structure [III] as suggested by Kapeller-Adler (1952).



[III]

Methods Employed: Purified hog kidney histaminase was prepared according to the method of Kapeller-Adler. Stomach histidine decarboxylase was prepared according to the method of Haakanson.

Histamine-^{3}H (side chain) was prepared from L-histidine-^{3}H (side chain-^{3}C) using bacterial histidine decarboxylase purified from *Cl. welchii* extracts.

The labeled compounds were incubated for various periods of time in the presence and absence of unlabeled substrates after preincubating the enzymes with pyridoxal phosphate. Standards were prepared by omitting the enzyme from the incubation mixture.

Labeled and unlabeled histamine and histidine were assayed using the procedures described in last year's annual reports. $^{3}H_2O$ water was determined using Thunberg tubes.

Major Findings: Incubation of histamine and histamine-^{3}H with purified histaminase resulted in the complete destruction of histamine and quantitative conversion of ^{3}H-label to $^{3}H_2O$. This result suggests that the mechanism proposed above is correct and that alternate mechanisms which do not involve the formation of a double bond in the side chain are incorrect.

When about 50% of the histamine was deaminated the quantity of $^{3}H_2O$ formed was exactly equivalent to the amount of histamine destroyed. Current work is concerned with applying this finding in the development of a specific assay for histaminase activity in animal tissues.

Incubation of L-histidine-^{3}H with the mammalian histidine decarboxylase did not lead to the formation of $^{3}H_2O$ indicating that decarboxylation of histidine does not proceed by the mechanism described above [II].

Significance to Bio-medical Research and the Program of the Institute: These studies provide valuable information on histamine metabolism which may be used for the search and design of drugs for the treatment of pathological conditions mediated by histamine.

414

Proposed Course of Project: A study of the DAO activity in normal and diseased tissues is proposed.

Honors and Awards: None

Publications: None

Serial No.-NHI-174
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A Specific and Sensitive Assay for Aminoguanidine: Its Application to a Study of the Distribution of Aminoguanidine in Rat Tissues

Previous Serial Number: NHI-371

Principal Investigators: Dr. M. A. Beaven, and Mr. John W. Gordon

Other Investigators: Dr. S. Jacobsen and Dr. W. B. Severs

Cooperating Units: Dr. W. B. Severs is a U.S.P.H.S. Postdoctoral Fellow.

Man Years:

Total:	0.7
Professional:	0.7
Other:	0

Project Description:

Objectives: To design a specific and sensitive assay for aminoguanidine and study the distribution and localization of this drug in animal tissues.

Methods Employed: A sample (1 ml) of perchloric acid homogenate of the tissue is mixed with concentrated HCl and 0.1 ml of 0.1% ethanolic solution of *p*-nitrobenzaldehyde and heated 100° for 5 min. The reaction mixture is made alkaline with NaOH and the yellow derivative of aminoguanidine is extracted into ethyl acetate. The ethyl acetate extract is mixed with heptane and 0.1 N HCl and the derivative extracted into the acid phase. Standards are prepared by taking known amounts of aminoguanidine through the procedure and tissue blanks are prepared using homogenates of tissues taken from nontreated animals. The acid extract is made alkaline with NaOH and the optical density determined at 380 mμ.

Major Findings: The optical density reading was found to be linearly related to the amount of aminoguanidine over the range 0.1 to 10 μg. The values for standards were reproducible from day to day. Values for tissue blanks were from 0.010 to 0.030 (optical density units) compared to readings of 0.140 for 1 μg of aminoguanidine. The blank values can be reduced to less than 0.010 by washing the ethyl acetate extract once with 0.1 N NaOH, before extracting the derivative back into dilute HCl. Therefore the procedure is satisfactory for the assay of 0.1 to 1.0 μg of aminoguanidine.

Chromatographic studies showed that the colored product obtained from tissue homogenates was derived solely from aminoguanidine. Hence, metabolites, if present, did not interfere with the assay.

The high sensitivity of the method is due to the complete absence of interfering material. The method was specifically designed to take advantage of the basic properties of the aminoguanidine-*p*-nitrobenzaldehyde derivative in contrast to the neutral Schiff's bases obtained by reacting amines with *p*-nitrobenzaldehyde.

Studies in rats showed that after the intravenous injection of aminoguanidine, the drug rapidly leaves the plasma and accumulates in various body tissue being particularly high in submaxillary gland, liver, and kidney. The compound was not detected in the brain. Thereafter the levels of drug slowly decline exponentially. The disappearance of drug cannot be accounted for by excretion into urine and feces. However, the metabolism of the drug has not been studied.

The drug is held in the tissue cells by an unknown process and is present largely in an unbound form in the supernatant fraction of the tissue homogenate

Presumably a mechanism, other than diffusion across a lipid membrane allows the rapid entry of drug into tissue cells, possibly a pH gradient or an active transport mechanism.

Significance to Bio-medical Research and the Program of the Institute: This assay permits studies on the distribution of aminoguanidine in tissues. Although this drug is a classical selective inhibitor of histaminase, and has been used for 15 yrs in studies on histamine *in vivo*, little is known of its distribution in the body.

Proposed Course of Project: This project will be terminated.

Honors and Awards: None

Publications: None

467

Serial No. -NHI-175
1. Chemical Pharmacology
2. Pharmacogenetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the rates of synthesis and degradation of lactate dehydrogenase (LDH) isozymes in rat heart and skeletal muscle

Previous Serial Number: NHI-332

Principal Investigators: Dr. Paul J. Fritz
Dr. Elliot S. Vesell

Other Investigators: None

Cooperating Units: Dr. Fritz is at the University of Alabama Medical Center, Department of Molecular Biology, Birmingham, Alabama

Man Years:

Total:	0.15
Professional:	0.15
Other:	0

Project Description:

Objectives: More than 200 enzymes have thus far been shown to exist in multiple molecular forms. Although investigation of the LDH isozymes has been more intensive than that of any other isozymic system, the initial problem of the biological function of 5 different intracellular enzymes all possessing ostensibly similar catalytic activity remains unsolved. Furthermore, the control and regulation of the isozyme patterns in different tissues of an animal are obscure. Differences in the isozyme patterns of a tissue could arise from differences in the rates of either synthesis and/or degradation or alternatively some regulation might exist at the ribosomal level, where assembly of the constituent inactive monomers into active tetramers presumably occurs.

Methods Employed: Rats received a protein hydrolysate uniformly labelled with C^{14} amino acids mixed with their ad lib. diet. The rats were sacrificed at various times and LDH-5 was isolated and purified from skeletal and cardiac muscle by DEAE chromatography and precipitation with antibody prepared to rat LDH-5 in rabbits. The radioactive counts (DPM/mg LDH-5) in the antibody precipitated material were determined in skeletal and cardiac muscle by procedures and techniques similar to those employed by Berlin and Schimke for tryptophan pyrrolase and arginase (Molecular Pharmacology 1: 149-156, 1965). Considerations of non-LDH-5 radioactivity precipitating with the antibody and of the

differences in pool sizes of LDH-5 from skeletal and cardiac muscles were made and included in calculation of the results.

Major Findings: In cardiac muscle where LDH-1 predominates and little LDH-5 is found, the half-life for LDH-5 was 2 days whereas in skeletal muscle, where LDH-5 predominates, the half-life of LDH-5 was 40 days.

Significance to Biomedical Research and Program of the Institute: The turnover rate of LDH-5 varies in different tissues. It appears that this difference in decay is largely responsible for tissue specific isozyme patterns In tissues where large amounts of an isozyme exist, the turnover rate is prolonged, whereas the decay of an isozyme in low concentrations is more rapid, in accord with previous observations of Berlin and Schimke.

Proposed Course of Project: Antibodies will be made to LDH-1 and similar turnover studies performed. Also alterations of turnover rates of various isozymes in tumors and after hormone administration will be studied since under these conditions the LDH isozyme pattern is known to change.

Honors and Awards: For studies on LDH isozymes the Society for Experimental Biology and Medicine gave Dr. Elliot S. Vesell the 1967 Samuel James Meltzer Award for outstanding contributions to experimental biology and medicine.

Publications: None.

Serial No. -NHI-176 (c)
1. Chemical Pharmacology
2. Pharmacogenetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Mechanism of Drug-Induced Hemolysis and Methemoglobinemia in Glucose-6-Phosphate Dehydrogenase Deficiency

Previous Serial Number: NHI-329

Principal Investigators: Dr. Ian M. Fraser
Dr. Elliot S. Vesell

Other Investigators: None

Cooperating Units: Dr. Fraser is a Special Fellow, NIGMS, and is also on fellowship from School of Medicine, Loma Linda University, Loma Linda, California.

Man Years:

Total:	0.7
Professional:	0.7
Other:	0

Project Description:

Objectives: Extensive studies of the clinical and genetic aspects of drug-induced hemolysis have been published, particularly with regard to glucose-6-phosphate dehydrogenase (G-6-PD) deficiency. However, some pharmacological aspects of this problem remain obscure, particularly the precise mechanism of drug-induced hemolysis in G-6-PD deficient erythrocytes. The early studies by Brodie and Udenfriend indicated that a metabolite of pamaquine mediates methemoglobin formation and hemolysis. In the present study, we have compared the effects of these metabolites in normal and glucose-6-phosphate deficient erythrocytes. From this and subsequent work it is clear that hemolysis is caused not by the drug itself but by a metabolite of the drug.

Methods Employed: In addition to measurement of hemolysis produced by various agents in normal and G-6-PD deficient erythrocytes, effects on osmotic fragility and mechanical fragility were studied. To elucidate some of the biochemical changes which precede hemolysis, methods have been employed for the measurement of methemoglobin, reduced glutathione, and oxidized and reduced pyridine nucleotides. Various drug metabolites with oxidant properties were obtained synthetically or from the urine of dogs receiving antimalarials.

470

Major Findings: Rapid and complete stoichiometric depletion of reduced glutathione accompanies methemoglobin formation by the drug metabolites studied to date: 8-amino-5,6-quinolinediol, 5,6-dihydroxypentaquine, p-aminophenol and nitrosobenzene. However, in vitro only small effects on osmotic fragility accompanied these changes.

Hydroxylated metabolites of primaquine and acetanilid increased the mechanical, but not the osmotic, fragility of G-6-PD deficient human erythrocytes more than that of normal erythrocytes. These metabolites generally decreased glutathione content and increased methemoglobin content more markedly in G-6-PD deficient than in normal erythrocytes. Parent drugs were inactive at the same concentrations. Mechanical fragility is suggested as a useful technique for testing drug metabolites in vitro for hemolytic actions that they might exert in vivo. It may also serve to investigate the mechanism by which drug metabolites exert oxidant effects on the erythrocyte membrane.

Significance to Biomedical Research and Program of the Institute: A large number of individuals of various genotypes in this country and abroad possess erythrocytes deficient in G-6-PD and hence are susceptible to serious side effects from drug-induced hemolysis. In addition to its intrinsic pharmacological interest, investigation of the mechanism of hemolysis may contribute to the improved management of certain types of drug therapy in susceptible patients. Furthermore, this study has suggested the usefulness of mechanical fragility as an in vitro test system for such drugs and their metabolites, enabling assessment of these agents before the occurrence of adverse reactions to them.

Proposed Course of Project: Study of additional biochemical changes preceding hemolysis is planned and a continued evaluation of the factors responsible for drug-induced hemolysis in normal and G-6-PD deficient erythrocytes is planned.

Honors and Awards: None.

Publications:

Fraser, Ian M. and Vesell, Elliot S.: Effects of metabolites of primaquine and acetanilid on normal and glucose-6-phosphate dehydrogenase deficient erythrocytes. J. Pharmacol. and Exptl. Therapeutics (in press).

Fraser, Ian M. and Vesell, Elliot S.: Effects of drugs and drug metabolites on erythrocytes from normal and glucose-6-phosphate dehydrogenase deficient individuals. Annals of the New York Academy of Sciences, Conference on Pharmacogenetics (in press).

Serial No. -NHI-177 (c)
1. Chemical Pharmacology
2. Pharmacogenetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Genetic control of drug levels in man

Previous Serial Number: None

Principal Investigators: Dr. Elliot S. Vesell
Dr. John G. Page

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: It is known that there are individual variations in the metabolism of many drugs in man. The objective of this study is to establish whether such differences in a healthy population are due primarily to environmental or genetic causes.

Methods Employed: We employed 14 pairs of volunteer twins from the Greater Washington area, of which 7 were identical and 7 nonidentical. All were Caucasian, over 21 years of age and in good health. None took drugs for at least one month before phenylbutazone administration. Each was typed for approximately 30 blood groups to document the nature of the twinship; the results confirmed the twins' view of whether they were identical or nonidentical.

Each twin received at 8:30 a.m. a single oral dose of phenylbutazone as "Butazolidin" tablets from Geigy. The dose was approximately 6 mg/kg. Since only 100 mg tablets were available, dosages were adjusted to the closest 50 mg. Because the dose was not in all cases exactly 6 mg/kg, initial levels of plasma phenylbutazone were not exactly comparable in different individuals. However, in this study absolute plasma levels were not critical because within the levels attained, they did not affect the values for phenylbutazone half-lives. Twenty-four, or in some cases 48, hours after phenylbutazone administration blood was drawn in tubes containing oxalate, and the plasma assayed for phenylbutazone by the method of Burns et al. (J. Pharmacol. exp. Ther. 109: 346, 1953). Subsequent specimens were drawn at regular intervals. To determine

4-72

whether administration of a single dose of phenylbutazone changed the rate of elimination of a subsequent dose, two sets of identical twins received a second similar dose of phenylbutazone 9 days after the first dose.

Antipyrine (18 mg/kg) and dicumoral (4 mg/kg) were also given in single oral doses to the same twins at intervals of two months following each other to avoid the possibility of induction. Blood levels were determined by the methods of Brodie, Axelrod, Soberman and Levy, and of Axelrod, Cooper and Brodie, respectively.

Major Findings: For each of these three drugs, intratwin differences were significantly less for identical than for fraternal twins. These results suggest that the large individual variations observed are determined primarily by genetic rather than environmental factors. In the individuals under study there was a six-fold range in the values for half-lives of phenylbutazone, a three-fold range for the half lives of antipyrine and a twelve-fold range for the half-lives of dicumarol. Each each drug, certain twins received at appropriate intervals a second identical dose of the same drug to determine the stability of the drug half-life. The drug half-life determined on the second administration was very close to that found initially. Therefore, in individuals not receiving additional drugs, the half-lives of phenylbutazone, antipyrine and dicumarol were stable traits.

For each drug, calculation of the contribution of heredity to the trait under question was made from the formula:

$$\frac{\text{variance within pairs of fraternal twins - variance within pairs of identical twins}}{\text{variance within pairs of fraternal twins}}$$

This formula permits a range of values from 0, suggesting negligible contribution of heredity, to 1, indicating strong hereditary influence. Variance within pairs was calculated from the formula

$$\frac{\Sigma \text{difference between twins}^2}{2n}$$

For each of the three drugs studied, the value for the contribution of heredity was 0.99, a value compatible with a very strong contribution by heredity to the trait under investigation.

It was found that there was no correlation in the individuals studied between the rate of decay for phenylbutazone and that for antipyrine.

Significance to Biomedical Research and Program of the Institute: Individual differences in drug metabolism of the magnitude described in these studies have therapeutic implications. Toxicity may develop primarily in subjects with long plasma half-lives, whereas those with short half-lives may not attain sufficient or sufficiently sustained levels to benefit from the drug. For phenylbutazone, antipyrine and dicumarol, variability among individuals

473

in the rate of drug elimination is genetically controlled. They represent a stable trait in humans not receiving other drugs. These observations suggest that determination of half-life in individuals given various compounds may permit greater therapeutic effectiveness. Although no correlation between the metabolism of phenylbutazone and antipyrine occurred, correlations between the rate of removal of these and other drugs from plasma will be sought, because such correlations might offer the therapeutic possibility that determination of the half-lives of certain drugs would indicate the rates at which other drugs are eliminated.

Proposed Course of Project: Other drugs, such as dilantin and hexobarbital, will be administered to twins to determine their half-lives and to test the more general applicability of the principles described. Studies in twins on the genetic control of the induction of drug-metabolizing enzymes will be initiated. The half-life of a drug such as dilantin would be determined before and after administration of several doses of the inducing drug phenobarbital to identical and fraternal twins. Differences between identical and fraternal twins in the extent of induction will be investigated.

Honors and Awards: Dr. Bernard B. Brodie was the Carl Wilhelm Scheele lecturer at the Royal Pharmaceutical Institute, Stockholm, Sweden, 1967.

Publications:

Vesell, Elliot S. and Page, John G.: Genetic Control of Drug Levels in Man: 1. Phenylbutazone. Science 159: 1479-1480, 1968.

474

Serial No. NHI-178 (c)
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Significance of Chlorpromazine Plasma Levels in Pharmacology and Clinical Medicine. I. Kinetics of Appearance and Disappearance of Chlorpromazine and Some of Its Metabolites in the Plasma of Psychiatric Patients

Previous Serial Number: NHI-336, NHI-339

Principal Investigator: Dr. Stephen H. Curry

Other Investigators: Dr. John H. L. Marshall
Dr. John Davis
Dr. D. Janowsky
Miss J. E. Derr

Cooperating Units: Dr. Marshall is a Staff Psychiatrist at St. Elizabeth's Hospital, Washington, D.C.
Drs. Davis and Janowsky are with the Adult Psychiatry Branch, NIMH

Man Years:

Total:	0.9
Professional:	0.4
Other:	0.5

Project Description:

Objectives: As part of an investigation into the nature and significance of plasma levels of chlorpromazine and its metabolites in psychiatric patients the kinetics of absorption, distribution in tissues, metabolism, and renal elimination of the compounds are being studied in man. An attempt is being made to understand the major factors affecting the plasma levels, and to determine means by which plasma levels can be controlled.

Methods Employed: Plasma samples from suitable patients are supplied by Drs. Marshall, Davis, and Janowsky. Chlorpromazine and its heptane soluble metabolites (chlorpromazine sulphoxide and the demethylated analogues of chlorpromazine) are determined in the plasma by the gas-chromatographic method described previously (see last year's reports).

Major Findings: 1) Chlorpromazine, chlorpromazine sulphoxide and the demethylated derivatives of chlorpromazine are all found in the plasma of chronically treated patients. However, of the metabolites, only chlorpromazin

475

in the rate of drug elimination is genetically controlled. They represent a stable trait in humans not receiving other drugs. These observations suggest that determination of half-life in individuals given various compounds may permit greater therapeutic effectiveness. Although no correlation between the metabolism of phenylbutazone and antipyrine occurred, correlations between the rate of removal of these and other drugs from plasma will be sought, because such correlations might offer the therapeutic possibility that determination of the half-lives of certain drugs would indicate the rates at which other drugs are eliminated.

Proposed Course of Project: Other drugs, such as dilantin and hexobarbital, will be administered to twins to determine their half-lives and to test the more general applicability of the principles described. Studies in twins on the genetic control of the induction of drug-metabolizing enzymes will be initiated. The half-life of a drug such as dilantin would be determined before and after administration of several doses of the inducing drug phenobarbital to identical and fraternal twins. Differences between identical and fraternal twins in the extent of induction will be investigated.

Honors and Awards: Dr. Bernard B. Brodie was the Carl Wilhelm Scheele lecturer at the Royal Pharmaceutical Institute, Stockholm, Sweden, 1967.

Publications:

Vesell, Elliot S. and Page, John G.: Genetic Control of Drug Levels in Man: 1. Phenylbutazone. Science 159: 1479-1480, 1968.

474

Serial No. NHI- 178 (c)
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Significance of Chlorpromazine Plasma Levels in Pharmacology and Clinical Medicine. I. Kinetics of Appearance and Disappearance of Chlorpromazine and Some of Its Metabolites in the Plasma of Psychiatric Patients

Previous Serial Number: NHI-336, NHI-339

Principal Investigator: Dr. Stephen H. Curry

Other Investigators: Dr. John H. L. Marshall
Dr. John Davis
Dr. D. Janowsky
Miss J. E. Derr

Cooperating Units: Dr. Marshall is a Staff Psychiatrist at St. Elizabeth's Hospital, Washington, D.C.
Drs. Davis and Janowsky are with the Adult Psychiatry Branch, NIMH

Man Years:

Total:	0.9
Professional:	0.4
Other:	0.5

Project Description:

Objectives: As part of an investigation into the nature and significance of plasma levels of chlorpromazine and its metabolites in psychiatric patients the kinetics of absorption, distribution in tissues, metabolism, and renal elimination of the compounds are being studied in man. An attempt is being made to understand the major factors affecting the plasma levels, and to determine means by which plasma levels can be controlled.

Methods Employed: Plasma samples from suitable patients are supplied by Drs. Marshall, Davis, and Janowsky. Chlorpromazine and its heptane soluble metabolites (chlorpromazine sulphoxide and the demethylated analogues of chlorpromazine) are determined in the plasma by the gas-chromatographic method described previously (see last year's reports).

Major Findings: 1) Chlorpromazine, chlorpromazine sulphoxide and the demethylated derivatives of chlorpromazine are all found in the plasma of chronically treated patients. However, of the metabolites, only chlorpromazin

sulphoxide is found in quantities comparable with those of chlorpromazine. Occasionally, demonomethylchlorpromazine sulphoxide is observable. In view of the lack of significant pharmacological activity characteristic of chlorpromazine sulphoxide, and the relatively insignificant quantities of the other compounds, it is probable that among the n-heptane-extractable materials, only the parent compound contributes to the pharmacological effects. In acutely treated patients, chlorpromazine itself is the only compound observable.

2) Comparison of chlorpromazine plasma levels, after oral and intramuscular administration of the drug to the same patient, indicates that a variable percentage (0% to 100%) of an oral dose is absorbed from the gastrointestinal tract into the blood stream (assuming that the level after intramuscular administration represents 100% absorption). The low plasma levels after oral administration could have been caused by complete absorption, followed by rapid elimination, involving only partial circulation, but since the half-lives after the two doses were comparable and long, this seems unlikely. The conclusion of poor absorption is in direct conflict with the established concept that chlorpromazine is "rapidly and completely absorbed".

3) The reasons for the variable absorption of chlorpromazine are not known, and experiments with fasted patients, and liquid and tablet preparations have failed to reveal oral administration conditions under which the plasma levels of the drug after oral and intramuscular administration to the same patient are comparable. Further evidence for the variable absorption of chlorpromazine is provided by measurements of the "apparent volume of distribution" of the compound. This figure is reliably determined as approximately 7X body weight after intramuscular administration, but varies from 7 to 70X body weight after oral administration, in those patients in whom the plasma levels are high enough for its determination. A high value indicates low absorption in comparison with the maximum value of 7.

4) A complicating factor in the above conclusions is the fact that plasma levels of chlorpromazine after intramuscular injection are often quite erratic showing rising levels at times such as 6 hours and 24 hours after the injection. These rises often occur during exercise involving the injection site, and they may indicate release of bound chlorpromazine from the site of injection or they may indicate other forms of irregular drug distribution. Hence absorption may not be rapidly complete even after intramuscular administration, and a true figure for the apparent volume of distribution of chlorpromazine in man will only be determined after intravenous administration.

5) Graphs of % absorption against time, (% of total absorption occuring in any time period) indicate that almost all the absorption taking place after oral administration occurs within 3 hours of dosage. The remaining chlorpromazine is presumably eliminated in faeces.

6) Urinary excretion of unchanged chlorpromazine accounts for elimination of not more than 1% of the dose. Urinary excretion occurs to a greater extent in patients with urinary pH values below 6 than in patients with higher urinary pH values. By calculating the amount of drug absorbed from the values

determined for the apparent volume of distribution, it has been shown that the percentage of drug absorbed which is eliminated in urine varies from 0-1% and is directly dependent on urinary pH.

7) Variations in plasma levels in comparable patients have been shown to be considerable. Thus, in chronically treated patients, wide interpatient variation has been observed in pre-dosage levels (0-770 ng/ml), in the rise in levels after dosage (16-225 ng/ml), in the percentage increase in plasma levels after dosage (3-300%) and in the half-life of elimination in the period after the first two hours (1.9 to 31.0 hours, although in 90% of patients the half-life was 6.0 hours or less). The plasma level at any one time results from any one of a number of possible combinations of residual level, degree of absorption, and rate of elimination.

8) Plasma levels have been shown to be predictably affected by dose, within the limits of individual patients. Thus, when patients are treated with, respectively, 100, 200 and 300 mg doses of chlorpromazine, the peak plasma level varies in proportion to the dose, and the peak time varies little.

9) A patient stabilized on a 200 mg b.d. dosage regimen, showing reproducible drug plasma level curves over a period of several weeks, was treated with 30 mg phenobarbital each night for three weeks concurrently with the chlorpromazine. During this time, the plasma levels of chlorpromazine dropped, and the rate of elimination increased. On cessation of phenobarbital treatment, the plasma levels steadily rose and the rate of elimination returned to the previous level. These results provide evidence of the importance of stimulators of drug metabolism (such as phenobarbital) in determining plasma levels of drugs.

10) The rapid rate of elimination of chlorpromazine determined contradicts the established concept that chlorpromazine persists in the body for a long period of time after cessation of dosage. This concept has been based on determinations by non-selective analytical methods, which detect metabolites as well as the parent drug. It is probable that water soluble metabolites do in fact persist for long periods of time.

11) Binding of chlorpromazine to human plasma proteins has been determined by equilibrium dialysis 95% of the chlorpromazine content of plasma is bound and very little interpatient variation has been detected.

Significance to Bio-Medical Research and the Program of the Institute: A knowledge of the levels of active materials present in plasma of patients undergoing treatment with chlorpromazine could be of vital importance in the design of optimum therapy procedures. In the future it is possible that patients will be dosed according to their particular rate of metabolism, permitting the design of a regimen with a dose frequency rather than magnitude suitable for the maintenance of the desirable plasma level. The current project is an examination of this possibility.

477

Proposed Course of Project: Other factors affecting plasma levels will be studied. Methods for the analysis of other chlorpromazine metabolites will be devised. Dosage regimens based on rates of metabolism of particular patients will be calculated and tested. Sustained release preparations of chlorpromazine will be studied.

Honors and Awards: Dr. Bernard B. Brodie received the 1967 Albert Lasker Award for Basic Medical Research

Publications: None

Serial No. NHI-179
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Significance of Chlorpromazine Plasma Levels in Pharmacology and Clinical Medicine. II. Tissue Localization of Chlorpromazine and its Metabolites in Animals and Man

Previous Serial Number: NHI-336, and NHI-339

Principal Investigator: Dr. Stephen H. Curry

Other Investigators: Dr. Harriet M. Maling
Miss Julia E. Derr
Mrs. Martha A. Williams

Cooperating Units: None

Man Years:

Total:	0.8
Professional:	0.4
Other:	0.4

Project Description:

Objectives: As part of an investigation of the nature and significance of plasma levels of chlorpromazine in humans, experiments are in progress to determine: (1) whether the drug and/or its metabolites are preferentially localized in particular tissues; (2) whether tissue levels and plasma levels decline with similar half-lives; (3) which metabolites accumulate in the body, and which merely facilitate elimination of chlorpromazine; (4) plasma protein binding and the "apparent volume of distribution" of chlorpromazine and its metabolites; (5) inter- and intra-species comparability of these phenomena.

Methods Employed: Biological material is collected and homogenized by standard procedures. Chlorpromazine and its heptane extractable metabolites are determined by the gas-chromatographic procedure described previously. Combined chlorpromazine and metabolites are determined by liquid scintillation counting of tissue homogenates from animals treated with S^{35} labeled chlorpromazine. Chlorpromazine and all of its metabolites are determined by liquid scintillation counting of samples from extracts obtained as follows:

479

Extract No.	Procedure	Compounds Evaluated
I	Whole homogenate	Chlorpromazine and all of its metabolites.
II	Heptane extract from plasma + NaOH	Chlorpromazine, its demethylated derivatives, and their sulphoxides
II'	The heptane extract in II, after washing with an equal volume of buffer solution pH 4.6	Chlorpromazine in the Heptane Heptane extractable metabolites in the aqueous layer
III	Ether extract from the aqueous residue in II, after adjustment of the pH to 7	Ether extractable material tentatively identified as 7-hydroxychlorpromazine and its analogues
III'	Aqueous residue in III	Water soluble metabolites, e.g. glucuronides

Major Findings: 1) Three fractions of chlorpromazine-like material are present in the tissues of rats after intravenous or intraperitoneal injection of chlorpromazine. They are: a) the heptane extractable materials (chlorpromazine, the demethylated analogues of chlorpromazine and their sulphoxides); b) ether soluble materials, and c) water soluble materials. In general, all of the heptane extractable radioactivity is accountable as chlorpromazine, chlorpromazine sulphoxide and demonomethylchlorpromazine. The ether soluble material is amphotetic in nature, as shown by the fact that it is extractable from ether into both basic and acidic solutions, but not into neutral aqueous solutions. Within the limits of known metabolites of chlorpromazine, this material can only be 7-hydroxychlorpromazine or a mixture of this compound with its own demethylated or sulphoxidized analogues. The water soluble material is considered to be the known conjugates of chlorpromazine metabolites - glucuronides and sulphate esters.

2) All three of the above fractions have been detected in brain, caudate nucleus, liver, muscle and plasma of rats pretreated with chlorpromazine. Over the time period 2-24 hours following intraperitoneal injection, tissue and plasma levels of each fraction are in equilibrium, as shown by near constant tissue:plasma concentration ratios. The lipid soluble materials, such as chlorpromazine, chlorpromazine sulphoxide and demonomethylchlorpromazine have high tissue:plasma ratios, indicating extensive localization of these compounds at tissue binding sites. The ether-soluble and water-soluble materials are distributed with tissue:plasma ratios less than 1, suggesting that these materials possibly penetrate tissues only by their presence in capillary circulation. This hypothesis will require plasma protein binding studies with all the metabolite fractions for adequate testing. There is no evidence for long-term persistence of non-reversibly bound radioactivity in rat tissues.

3) Chlorpromazine is highly bound to plasma proteins of rats, rabbits, dogs, and man. When added to blank plasma of these species, S^{35} chlorpromazine rapidly equilibrates reversibly with protein binding sites, and equilibrium dialysis has shown that in rats, rabbits, dogs and man, 10.4%, 5.3%, 4.1% and 3.0% respectively of the plasma content is unbound. These variations could result from interspecies variations in the protein content of plasma. There is very little variation in these figures over the chlorpromazine concentration range 10-1000 ng/ml., and very little variation between separate individuals within one species. Specificity experiments have shown that the molecular species unbound is chlorpromazine and is not a radioactive impurity in the chemical. Addition of S^{35} chlorpromazine to plasma from psychiatric patients undergoing chlorpromazine treatment, and measurement of the specific activity of the chlorpromazine bound and unbound, by gas-chromatography and liquid-scintillation spectrometry, has shown that added chlorpromazine rapidly equilibrates with endogenous chlorpromazine in plasma.

4) The "apparent volume of distribution" of chlorpromazine in rats, rabbits and dogs has been determined by dividing the dose (mg/kg) of chlorpromazine by the calculated plasma level (μg/g) of the drug at to following intravenous or intraperitoneal administration. The corresponding figure for man has been determined in a similar way after acute intramuscular and oral doses, and after chronic oral doses, with a correction in this case for the residual level of chlorpromazine at t_o resulting from the doses previous to the experimental treatment. After both intravenous and intraperitoneal injection in rats, the "apparent volume of distribution" of chlorpromazine was found to be 21X body weight. The corresponding figures for rabbits and dogs after intravenous administration were 15 and 10.5 respectively. In man, after intramuscular administration, the figure was determined as 7X body weight, and after oral administration the range was 7-70X body weight.

Since similar plasma levels after different modes of administration indicate comparable degrees of absorption, and since, after intramuscular administration, in normal conditions, chlorpromazine absorption should be complete, the range of values obtainable after oral administration to humans probably indicates a variable degree of absorption, over the range 10-100% of the dose. It is noteworthy that the apparent volume of distribution in different species varies conversely with the degree of plasma protein binding and it is possible that the various species have similar ratios for tissue level:level in plasma water, and vary only in the extent of plasma protein binding of the drug.

Significance to Bio-medical Research and the Program of the Institute: In determining the significance of plasma levels of drugs in humans, it is essential to know whether plasma levels are proportional to levels of drugs at sites of action, and to determine any intra-species differences in such phenomena. Determination of tissue levels are an essential first step in such an investigation.

Proposed Course of Project: The significance of the various metabolite fractions in pharmacology will be determined. The distribution investigation will be continued to determine whether the "apparent volume of distribution" varies with such factors as dose and frequency of treatment.

Honors and Awards: None

Publications: None

Serial No. NHI- 180 (c)
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Significance of Chlorpromazine Plasma Levels in Pharmacology and Clinical Medicine. III. Relationships Between Pharmacological Responses and Plasma Levels in Man

Previous Serial Numbers: NHI-336, NHI-339

Principal Investigator: Dr. Stephen H. Curry

Other Investigators: Dr. John H. L. Marshall
Dr. John Davis
Dr. David Janowsky
Miss Julia E. Derr

Cooperating Units: Dr. Marshall is a Staff Psychiatrist of St. Elizabeth's Hospital, Washington, D. C.
Drs. Davis and Janowsky are with the Adult Psychiatry Branch, NIMH

Man Years:

Total:	0.4
Professional:	0.2
Other:	0.2

Project Description:

Objectives: As part of an investigation of the nature and significance of plasma levels of chlorpromazine and its metabolites in psychiatric patients, concurrent plasma level determinations and psychopharmacological examinations are being conducted in acutely and chronically treated patients.

Methods Employed: Chronically treated patients are evaluated for sedative, antipsychotic and absence of effects of chlorpromazine, and for side effects. Plasma level determinations of chlorpromazine are made with the gas-chromatographic procedure described earlier (see last years reports). Acutely treated patients are examined in similar ways, but additionally, blood pressure determinations are made.

Major Findings: (1) Chronically treated patients absorb and metabolize chlorpromazine at widely varying rates (see separate report). As the result of this, the plasma levels of the drug vary widely within any patient population receiving a standardized dosage regimen. Each patient, because of his particular rates of intake and elimination of the drug achieves a characteristic peak plasma level within 3 hours of an oral dose, and 80% of

patients have relatively low levels (10% of peak) by the time that the next dose is due. Among such populations, a number of patients have been found to be unresponsive to the drug, and such patients have undetectable plasma levels, indicating either minimal absorption or very rapid metabolism.

(2) Patients considered to be responding well to chlorpromazine have been found to require a peak plasma level of 50-500 ng/ml of the drug. Because of the nature of schizophrenia, it has not been possible by patient observation alone to detect differences in response at different plasma levels within this range.

(3) One patient considered to be erratic in her response to chlorpromazine, as shown by alternating acutely schizophrenic and drug controlled states, was shown to be erratic also in her plasma levels of the drug.

(4) Drug-induced sedation was observed in one particular chronically treated, well-controlled patient, by increasing the dose (and hence the plasma levels) by 50%. This effect was observable on the first day of the new dosage regimen, and peak sedation occurred at the peak plasma level. A reduction of the original dose and plasma level of the drug in this patient caused an acute schizophrenic attack on the first day of the reduced dosage regimen, after which the original dosage regiment was re-established. These effects were repeatable. When a procedure of this kind was carried out in a patient with peak plasma levels of chlorpromazine below 50 ng/ml., very little change in the response was detectable, possibly indicating that this patient was receiving doses of chlorpromazine too low to cause pharmacological effects, and that this patient, who was responding quite well to treatment, was responding more to environmental factors, than by pharmacological mechanisms.

(5) Most acutely treated patients show some sedation after the first dose of chlorpromazine, even though the peak plasma levels of the drug are in the range 20-100 ng/ml. The sedation is most severe at the peak level. Hypotension is also common in such patients, and in particular the tendency for postural hypotension is very strong. These responses are greatest at peak plasma levels, and occur at levels much lower than those tolerated without such effects in chronically treated patients. Thus there is strong evidence for the existence of a form of tolerance other than the more rapid metabolism of the drug expected to occur after prolonged exposure.

(6) Extrapyramidal side effects have not been observed to date in any of the patients studied. In chronically treated patients, such effects are known to occur after doses higher than those causing sedation, and hence the effects are not seen in patients with dosage regimens designed to prevent the occurrence of sedation. Extrapyramidal effects are not usually observable after acute doses.

484

(7) Plasma level studies have provided strong evidence for the existence of a previously ill-defined side-effect of chlorpromazine. One chronically treated patient, receiving 950 mg. b.d., and not sedated, was found to have concentrations of chlorpromazine in her plasma varying from 650-850 ng/ml. In spite of this the patient was unresponsive to the drug and was uncooperative to all forms of treatment. On reduction of the dose by 30% and reduction of the plasma levels to below 500 ng/ml., the patient became much more cooperative. This provides evidence for the existence of some form of chlorpromazine induced psychotoxicity, seen as an aggravation of schizophrenia.

Significance to Bio-medical Research and to the Program of the Institute: As the result of controlled studies of the relationship of effects to plasma levels, drug concentrations may become known at which more predictable pharmacological effects can be obtained. If this is found to be so, then the immense variability of effects observable with chlorpromazine will be controllable by means of more rational regimens of dosage.

Proposed Course of Project: The above findings, and other relationships between levels and effects, will be replicated as much as possible. In particular, it is anticipated that studies of plasma level and patient performance in psychological evaluation tests will be possible. When desirable plasma levels and effects are known, then experiments with controlled plasma levels will become valid. To date, it seems likely that monitoring of patients during chlorpromazine treatment could be of value for at least three purposes: (1) detection of non-absorption of chlorpromazine; (2) detection of psychotoxicity of chlorpromazine; (3) determination of whether the apparent schizophrenic state in a patient is sensitive to chlorpromazine, by concurrent modification of the dose and plasma level of the drug and careful observation of effects. In each of these cases, plasma level monitoring would lead to a reduction in unnecessary exposure of patients to the drug, and hence to possible chronic side effects.

Honors and Awards: None

Publications: None

485

Serial No. NHI-181 (c)
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Plasma Levels of Thioridazine (Mellaril) in Psychiatric Patients

Previous Serial Number: None

Principal Investigator: Dr. Stephen H. Curry

Other Investigator: Dr. John H. L. Marshall

Cooperating Unit: Dr. Marshall is a Staff Psychiatrist at St. Elizabeth's Hospital, Washington, D. C.

Man Years:

Total:	0.05
Professional:	0.05
Other:	0

Project Description:

Objectives: (1) To devise a method for the routine analysis of thioridazine in body fluids from animal and human sources; (2) to study the kinetics of absorption, tissue localization, and elimination of the drug in animals and man; and (3) to determine the relationship, if any, between responses in man and the physiological disposition of the compound.

Methods Employed: Plasma samples from psychiatric patients are supplied by Dr. Marshall. Thioridazine is extracted from alkalinized plasma (5 ml) into n-heptane (10 ml) containing 1.5% isoamyl alcohol, and back extracted into 0.05 N HCl (2 ml). The aqueous phase followed by made alkaline and the thioridazine is extracted into a fresh heptane sample (50 ml). Thioridazine is determined in the final heptane extract by gas chromatography (GLC), using an argon ionization detector.

Major Findings: (1) Thioridazine has a retention time of 3 1/2 min on an OV-17 (Supelco, Inc.) GLC column at 275°. (2) The minimum sample detectable is 30 ng. (3) With the extraction procedure described above, the recovery of thioridazine is >80%, blanks are negligible. Thioridazine in a 5 ml plasma sample can be determined at concentrations as low as 30 ng/ml. (4) In two patients undergoing chronic treatment with 100 and 300 mg doses of thioridazine b.d., plasma levels two hours after a morning dose were 244 and 1800 ng/ml respectively. (5) No metabolites of thioridazine were detected in the heptane extracts.

486

Significance to Bio-medical Research and the Program of the Institute: The determination of the kinetics of absorption and elimination of psychotherapeutic drugs in man is important to the understanding of the variability of response to such drugs and to the design of dosage regimens suitable for individual patients.

Proposed Course of Project: Thioridazine is steadily displacing chlorpromazine as the phenothiazine derivative of choice in many psychiatric hospitals, because of its lower sedative, extrapyramidal and hypotensive effects. For this reason, a program of experiments similar to those described for chlorpromazine (see other reports) is planned.

Honors and Awards: None

Publications: None

Serial No.-NHI-182
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of Drugs on Protein Synthesis

Previous Serial Number: None

Principal Investigators: Dr. Arthur K. Cho

Other Investigators: Mrs. Barbara E. Hodshon

Cooperating Units: None

Man Years:

Total:	0.8
Professional:	0.4
Other:	0.4

Project Description:

Objectives: As part of a general study investigating the toxicological properties of drugs, the effects of phenylbutazone on protein synthesis was examined. Other workers have described the inhibiting effect of anti-inflammatory agents on amino acid incorporation in reticulocytes, lymphocytes, and liver microsomes. The objective of this research was to examine the structural requirement for the inhibition and to elucidate the mechanism of this effect.

Methods Employed: Standard biochemical techniques were employed.

Major Findings: Initially, rats were given i.p. doses of 100 mg/kg of phenylbutazone, the liver microsomes isolated and examined *in vitro* for amino acid incorporating activity. At 2-3 hours after treatment, the incorporation of C^{14} leucine into the microsomes was depressed, from 30 to 90% of control in six experiments. To minimize the variability of the effects and to examine the effect at a biochemical level *in vitro* experiments were performed with the drugs added to the incubation mixture. Under these conditions phenylbutazone, at concentrations of 2-4 mM caused a 40-50% inhibition of amino acid incorporation into liver slices, liver microsomes and polyribosome preparations. While these concentrations were too high to be likely to reflect the immediate *in vivo* effects, they were examined further for their role in the toxicity of the compound. This inhibition was found to be very general in its structural requirements and several compounds differing from phenylbutazone only in the side chain substitution at position 4 were comparable in their potency although increased hydrocarbon characteristics appeared to

488

enhance activity. Oxyphenbutzone, a metabolite of phenylbutazone, was about $^1/_3$ as effective while another metabolite in which the side chain was oxidized was inactive.

Since phenylbutazone is known to enhance the anticoagulant effect of warfarin, the action of warfarin on the ribosomal system was also examined to look for potentiation of the phenylbutazone effect. In addition, the anti-coagulants were tested in the Mizushima assay for anti-inflammatory action. This latter assay measures the ability of a compound to inhibit the heat coagulation of albumin, and has been shown to give a positive response to anti-inflammatory agents but not to analgetic-antipyretic agents. Phenyl-butazone and warfarin exhibited additive effects in both systems. These actions, and the comparable abilities of both classes of compounds to uncouple oxidative phosphorylation suggests common structural features exist in these molecules relating them to each other and to these effects. Indeed, these and other anti-inflammatory, and anticoagulant compounds are weakly acidic, and have an aromatic ring connected to a lipophilic structure such as a hydrocarbon or another aromatic ring by 2 or 3 atoms of high electron density.

Significance to Bio-medical Research and the Program of the Institute: The potentiation of the pharmacological and toxicological effect of one drug by another therapeutically unrelated drug is a well known phenomenon. These experiments suggest that this potentiation could result from a additive effect by two chemically related drugs whose pharmacological relationship may not be obvious. Experiments like this may aid in predicting this type of potentiation.

Proposed Course of Project: Future experiments will attempt to determine the mechanism by which these drugs are affecting amino acid incorporation.

Honors and Awards: None

Publications: None

489

Serial No.-NHI-183
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Microsomal N-Hydroxylation System

Previous Serial Number: None

Principal Investigators: Dr. Arthur K. Cho

Other Investigators: Mrs. Barbara E. Hodshon

Cooperating Units: None

Man Years:

Total:	0.4
Professional:	0.2
Other:	0.2

Project Description:

Objectives: The microsomal enzyme system in the liver is capable of converting aromatic amines to their N-hydroxy derivatives. The resulting aromatic N-hydroxylamines have been postulated to be the active agents in toxic reactions such as methemoglobinemia that are associated with the parent amines. The objective of this study is to characterize the enzyme or enzymes responsible for this conversion in terms of substrate specificity, induction by other compounds and inhibition.

Previous work involved the development of an assay method for the conversion using C^{14}-labeled aniline as the substrate and extraction of nitrosobenzene, and oxidized derivative of the product into heptane for counting. Experiments were performed to determine the Michaelis Constant, Km, for the enzyme.

Methods Employed: Standard biochemical and radiochemical techniques were used.

Major Findings: Experiments in which the Km was measured gave variable values dependant on substrate concentration ranges. At low substrate concentrations (0.042 to 1.33 mM) Km values of about 10^{-4} M were obtained, and at higher levels (0.55 to 16.66) values of about 15×10^{-3} were obtained. This data was consistent with the postulated existence of 2 enzymes or enzyme systems capable of effecting the N-hydroxylation of aniline.

490

A possible means of examining components of this oxidation reaction is to selectively inhibit one of the enzyme systems oxidizing aniline, and experiments were performed to evaluate this approach. Initially, the relationship of the N-hydroxylating system to the hydroxylating system was examined by simultaneously determining p-aminophenol and nitrosobenzene. The C-hydroxylation system showed evidence of saturation at about 1 mM and the N-hydroxylation system was saturable only at substrate levels of 7 mM. A comparison of several inhibitors in these two reactions showed a difference with iproniazid. Thus, while both hydroxylation reactions were inhibited by dichlorophenol (5 mM) and a detergent, Triton-X (2.3 mg/ml) only C-hydroxylation was inhibited by iproniazid (see table).

mM	mM	Ratio of inhibited rate (V_I) to control (V_C) V_I/V_C	
[S]	[Iproniazid]	N-OH	C-OH
2	1.0	1.25	0.83
2	10.0	1.16	0
2	50.0	1.01	0
20	1.0	1.11	0.93
20	10.0	.92	0
20	50.0	1.09	0

[The concentrations of substrate for these experiments were such that the higher value Km of N-hydroxylation was involved.]

To extend the scope of the reaction further, the oxidation of p-toluidine and 2,4,6-trimethylaniline was examined by measuring the absorbance of the -N=O (290 mμ) group in a heptane extract of the reaction mixture. While this method was not sensitive enough to permit kinetic measurements, the results indicated that toluidine was oxidized at 4 x the extent of either aniline or trimethylaniline.

In summary, the results from this study suggest the existence of at least 2 enzyme systems capable of oxidizing aniline and indicate that the C-hydroxylation reaction can be selectively inhibited by iproniazid. In addition, preliminary experiments suggest that toluidine may be a good alternate substrate for the N-hydroxylation system.

Significance to Bio-medical Research and the Program of the Institute: In contrast to other microsomal enzyme systems which detoxify foreign compounds, the N-hydroxylation system appears to enhance the toxicity of certain aromatic amines. Since many drugs are amines or amine derivatives, knowledge of the substrate requirements and other properties of this enzyme is an important aspect of drug toxicity.

491

Proposed Course of Project: The selective inhibition of the C-hydroxylation system will be exploited to gain insight into differences in C- and N-hydroxylation reactions. Different amines may have different affinities for the enzymes and may provide a means of isolating one of the N-hydroxylating enzyme systems. The recent availability of p-toluidine-C^{14} can be readily adapted to the system as an alternate substrate.

Honors and Awards: None

Publications: None

Serial No. -NHI-184
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Localization of Drug Molecules in the Submaxillary Gland, and the Significance of this Localization in the Salivary Secretion of Drugs

Previous Serial Number: NHI-332, NHI-337, NHI-338

Principal Investigators: Dr. Arthur K. Cho, and Dr. Stephen H. Curry

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.20
Professional:	0.20
Other:	0

Project Description:

Objectives: With the advent of the technique of radioautography, the extensive localization of many drugs in the submaxillary glands has been observed, but no attempts have been made to understand the processes causing this localization. The basic drugs investigated have included chlorpromazine, nicotine, mepiracaine, and guanethidine. Similar observations were made here in the course of studies of two other basic compounds, Catapres (Boehringer) and Ba 31531 (Ciba). The object of this study is to investigate the mechanisms of the localization of these two compounds and the significance of the mechanisms to other basic compounds.

Previous experiments (see last year's reports) have shown that both Catapres and Ba 31531 are highly localized in the gland, with tissue to plasma ratios of 26:1 and 16:1 respectively. Furthermore, the compounds are concentrated reversibly within the gland itself and not in saliva. *In vitro*, slice experiments have indicated that the localization is pH-dependent, since the slice to medium concentration ratios for the two bases were found to increase with increasing pH.

Methods Employed: Standard biological sampling techniques were used. The assay methods have been described previously.

Major Findings: (1) The dependence on pH of the uptake of the two bases into slices of submaxillary gland incubated in Krebs Ringer Solution implies that the uptake is governed by an equilibrium distribution between two phases

493

of different pH values, separated by a lipid membrane. This could be caused by an intracellular pH of <7.4. Experiments were therefore carried out to determine the intracellular pH by the technique of distribution of DMO.

(2) Initially, the intracellular pH determined was 6.89 *in vivo*, and 6.40 *in vitro* with slices. Because of this discrepency, and because of the considerable error which would be recorded in this figure if part of the DMO distribution was caused by reversible binding of DMO to protein, the binding of DMO to homogenates was checked at various pH values. Sufficient binding was found (12.5% in a 30% homogenate at pH 6.3) to cast doubt on the original figure for intracellular pH. This implied that, to determine accurately the true intracellular pH of submaxillary gland, a binding correction must be made in the *in vivo* DMO distribution data. This correction must be determined at the true intracellular pH.

(3) Catapres and Ba 31531 are extensively bound to submaxillary gland homogenates (47% and 21% respectively to a 100% homogenate at pH 7.4), and preliminary experiments have shown that this binding is also pH dependent. Thus, to determine whether pH effects and binding of the bases together account for all of the uptake, binding studies of these compounds at the intracellular pH are also necessary. Additional evidence for the influence of binding is provided by the fact that the intracellular pH values calculated from *in vivo* data for DMO and Catapres are 6.89 and 5.60 respectively. When the results of preliminary binding studies are used to correct these figures, they are found to be 5.55 and 5.79 respectively. Concurrence of these two figures would indicate that the true intracellular pH had been determined.

Significance to Bio-medical Research and the Program of the Institute: The localization of drugs in body tissues is important in both toxicological and pharmacological effects, since drugs usually exert their action at sites of highest localization. General principles for such localization based on physicochemical properties of molecules would be of value in predicting the tissue distribution of new drugs and the organs which such compounds might affect. It is noteworthy that the endogenous amine histamine is apparently localized in submaxillary gland by these processes (see histamine reports) and that this localization may be significant in the action of histamine on salivary secretion.

Proposed Course of Project: The binding of these substances to tissue protein will be evaluated with different techniques and at varying pH values.

Honors and Awards: None

Publications: None

Serial No. NHI-185
1. Chemical Pharmacology
2. Enzymes Drug Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the disposition and mechanism of action of thalidomide and related compounds

Previous Serial Number: NHI-317

Principal Investigators: Dr. David A. Blake
Dr. James R. Gillette

Other Investigators: None

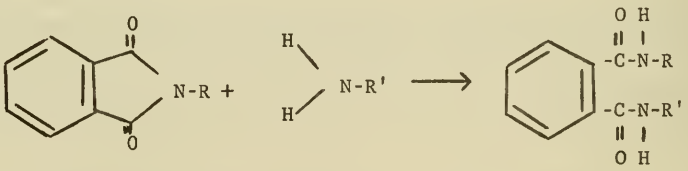
Cooperating Units: Dr. Blake is on the staff of the University of Maryland Pharmacy School

Man Years:

Total:	0.05
Professional:	0.05
Other:	None

Project Description:

Objectives: Although thalidomide is known to cause teratogenesis in laboratory animals, its mechanism of action remains unknown. Since thalidomide and its metabolites are derivatives of glutamic acid, it has been suggested that they might cause teratogenic effects by interfering with the metabolism of this amino acid. Alternatively, thalidomide may act by acylating essential substances in the fetus to form amides or esters; according to this mechanism, thalidomide and alkylating agents would cause teratogenic effects by similar mechanisms.



The purpose of this project is to evaluate possible mechanisms of thalidomide teratogenesis.

Methods Employed: Standard biochemical and physiological methods were employed.

495

Major Findings: Last year it was reported that thalidomide was metabolized more rapidly by rabbit liver than by rat liver or any other rabbit or rat tissues. During the past year we found that the enzyme which catalyzed the metabolism was probably an amidase localized in liver microsomes.

Significance to Biomedical Research and the Program of the Institute: These studies should provide a better understanding of the embryotoxic effects of thalidomide and related compounds.

Proposed Course of Project: The project was terminated.

Honors and Awards: None.

Publications:

Schumacher, H., Blake, D. A. and Gillette, J. R.: Modes of action of thalidomide. In Runner, M., Wilson, J. and Asling, C.W. (Eds.): Third Workshop in Teratology, in press.

Schumacher, H., Blake, D. A., Gurian, J. M. and Gillette, J. R.: A comparison of the teratogenic activity of thalidomide in rabbits and rats. J. Pharmacol. exp. Ther. 160: 189-200, 1968.

Schumacher, H., Blake, D. A. and Gillette, J. R.: Disposition of thalidomide in rabbits and rats. J. Pharmacol. exp. Ther. 160: 201-211, 1968.

496

Serial No. -NHI-186
1. Chemical Pharmacology
2. Enzymes Drug Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Pharmacological action, metabolism and tissue distribution of niridazole (ambilhar)

Previous Serial Number: None

Principal Investigators: Dr. Dennis R. Feller
Dr. James R. Gillette

Other Investigator: None

Cooperating Unit: Dr. Feller is an NIH Postdoctoral Fellow, NHI

Man Years:

Total:	0.25
Professional:	0.25
Other:	None

Project Description:

Objectives: Niridazole (1-[5-nitro-2-thiazoyl]-2-imidazolidinone) is an antischistosomal compound marketed in Europe and Africa. The clinical trials for the use of niridazole and related heterocyclic analogues resulted in the appearance of undesirable effects, including immunological reaction in about 10% of the patients treated. Preliminary in vitro studies by Keberle indicated that the drug is slowly absorbed from the G.I. tract, but rapidly removed from the blood. The principal metabolite in plasma, however, is irreversibly bound to plasma proteins. Studies with various tissues have revealed that the liver exhibits the greatest enzymatic activity and that the enzyme displays all the properties of nitroreductase. In this project the metabolism, tissue distribution and pharmacological actions of niridazole are studied in an attempt to elucidate the mechanism of irreversible binding of the drug to plasma proteins. These results should provide an insight into the mechanism of immunological reactions in man, presumably linked to the irreversible binding to proteins.

Methods Employed: Preparation and isolation of subcellular fractions of the rat liver followed standard procedures. The disappearance of niridazole from the various fractions was measured colorimetrically (400 mμ) following extraction with 2 volumes of ethylene dichloride. Niridazole, dissolved in acetone, was added to the incubation media in a final concentration of 0.8 mM.

497

Major Findings: 1) Initial experiments under a nitrogen atmosphere revealed (a) that niridazole is rapidly metabolized by the homogenate, 9000 x g and 78,000 x g supernatants in the presence of a NADPH-generating system, and (b) that the rate of disappearance was linear for approximately a 15-minute period. Studies with isolated microsomes in the presence of a NADPH-generating system did not show the properties as found with the other fractions since this fraction was unable to metabolize significant amounts of niridazole.

2) The presence of the nitro group suggested that nitroreduction may be a major pathway of drug elimination. However, the disappearance of niridazole remained unaffected by the gaseous atmosphere (nitrogen or air) during incubation with various subcellular fractions. In a series of experiments carried out in an atmosphere of either air or nitrogen, and in the presence or absence of an NADPH-generating system, it became apparent that niridazole is metabolized by at least two systems. Briefly, the removal of niridazole from the homogenate and 78,000 x g supernatant fractions was unaffected by the presence of NADPH, whereas the activity of the 9000 x g supernatant fraction was enhanced about two-fold by NADPH addition. These results indicate that there is a significant disappearance of niridazole, presumably due to protein binding, which is found in all fractions, with the possible exception of the isolated microsomes. It is evident that the enzymatic activity possessed by the 9000 x g fraction can be attributed to a NADPH-dependent enzyme system, apparently associated with the microsomes. Attempts to separate activities by dialysis or to identify the metabolite(s) by chromatography have thus far been unsuccessful.

Significance to Biomedical Research and to the Program of the Institute: Since antigens are usually of high molecular weight, it seems possible that the immunological side effects of niridazole may be mediated by irreversible binding of the drug to plasma protein. Such studies of the mechanisms of irreversible binding may be useful in elucidating the formation of antigens.

Proposed Course of Project: The availability of niridazole-C^{14} will be employed to determine the extent of tissue distribution and identification of possible metabolite(s) in vivo and in vitro. These data are necessary to correlate possible interrelationship between the metabolism and protein binding of this compound.

Honors and Awards: None.

Publications:

Gillette, James R.: Conference on the Pharmacological and Chemotherapeutic Properties of Niridazole and Other Antischistosomal Compounds. N. Y. Acad. Sci., New York, in press.

498

Serial No. -NHI-187
1. Chemical Pharmacology
2. Enzymes Drug Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the role of chlorpromazine in drug metabolis

Previous Serial Number: None

Principal Investigator: Dr. Dennis R. Feller
Dr. James R. Gillette

Other Investigator: None

Cooperating Unit: Dr. Feller is an NIH Postdoctoral Fellow, NHI

Man Years:

Total:	0.25
Professional:	0.25
Other:	None

Project Description:

Objectives: Many reports in the literature describe the stimulation of drug-metabolizing enzyme systems by prior treatment of animals with a variety of drugs. While it has been reported that pretreatment of rats with chlorpromazine (CPZ) and other phenothiazines increases benzpyrene hydroxylase activity in rat liver microsomes and accelerates the metabolism of a number of drugs in vivo, Dingell, in our laboratory, was unable to show that chlorpromazine enhances the metabolism of imipramine. In this project we investigated the effects of chlorpromazine pretreatment on the cytochrome P-450 content of liver microsomes and the metabolism of various types of substrates.

Methods Employed: N- and O-demethylase activities were determined by the measurement of formaldehyde production (Axelrod, Cochin) and the appearance of the product p-nitrophenol from p-nitroanisole, respectively. Cytochrome P-450 content was established by the method of Omura and Sato and the preparation of isolated hepatic microsomes followed standard procedures.

Major Findings: 1) From the results of several experiments employing rats (male, 50 ± 5 gm.) it was found that neither a single intraperitoneal injection of CPZ (25 mg/ml) nor three successive doses (25 hr apart) was capable of stimulating the cytochrome P-450 content in isolated rat liver microsomes. Experimental conditions were in accordance with the reported time and dosage schedules and strain of animal employed for the maximal response of enzyme induction with CPZ. In addition, there was an inability of CPZ pretreatment to enhance N-demethylase activity associated with the metabolism of ethylmorphine (remained unchanged in 2 of the 3 experiments). In the studies employing p-nitroanisole as a substrate, a consistent but barely detectable elevation in enzyme activity was observed by p-nitrophenol formation.

417

2) A previous paper from this laboratory (Smith et al., 1961) reported that CPZ in doses between 3-100 mg/kg activated the pituitary-adrenal system and elevated plasma corticosteroid levels for at least a 6-hour period. Since steroids are known to alter the activity of microsomal enzymes, the measurement of O- and N-demethylation after varying doses of chlorpromazine (0, 11, 33 and 66 mg/kg, i.p.) should change relative to control systems. Under these conditions, based on the results of three experiments, CPZ pretreatment failed to significantly alter the enzyme activities when measured four hours following the injection of CPZ. Although there was low enzyme activity associated with p-nitrophenol formation, the enzyme nevertheless remained unchanged by pretreatment with CPZ. In addition, cytochrome P-450 levels were found to be unaltered during this short period of drug exposure.

The inability to illustrate a consistent induction with the enzyme systems studied suggests that chlorpromazine may represent a stimulator of drug metabolism in vivo, which may not be directly mediated through an increased formation of cytochrome P-450 as previously reported for other polycyclic hydrocarbons.

Significance to Biomedical Research and to the Program of the Institute: This study should provide for a better understanding of the biochemical and physiological consequences of chlorpromazine-like compounds as therapeutic agents.

Proposed Course of Project: Studies will be taken in vivo and in vitro to measure the degree of interaction between CPZ, and/or steroids, and microsomal enzyme systems in the adult animal. A comparison of results obtained from studies with the immature and adult rats may be made for an evaluation of factors modifying the action of CPZ-like compounds.

Honors and Awards: None.

Publications: None.

500

Serial No.-NHI-188
1. Chemical Pharmacology
2. Enzymes Drug Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the rate-limiting step in drug oxidation by enzymes in hepatic microsomes

Previous Serial Number: None

Principal Investigator: Dr. J. R. Gillette

Other Investigators: Dr. Theodore E. Gram
Dr. Philippe L. Gigon
Dr. Jordan L. Holtzman
Dr. Donald S. Davies

Cooperating Units: Dr. Gigon receives a fellowship from the Swiss Academy of Medical Sciences

Drs. Gram and Holtzman are Research Associates in the Pharmacology-Toxicology Training Program, NIGMS

Man Years:

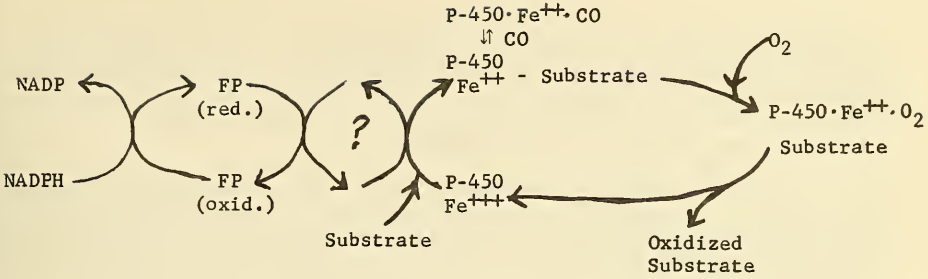
Total:	1.0
Professional:	1.0
Other:	None

Project Description:

Objectives: There is considerable evidence that the oxidation of various drugs and hydroxylation of steroids by NADPH-dependent enzymes in liver microsomes are mediated by cytochrome P-450. The mechanism of these enzyme reaction remains obscure. It has been postulated that NADPH reduces cytochrome P-450 either directly or indirectly through NADPH cytochrome c reductase, and that the reduced cytochrome P-450 reacts with oxygen to form an "active oxygen" complex which in turn oxidizes the drug or steroid. However, the finding that various substrates caused changes in the visible absorption spectrum of liver microsomes, even in the absence of NADPH, suggested that substrates formed complexes with the oxidized form of cytochrome P-450 and that the rate-limiting step of the overall reaction might be reduction of the substrate cytochrome P-450 complexes.

The sequence described above may be represented as follows:

501



The present work is oriented toward delineation of the rate-limiting step in this sequence and an understanding of its relationship to drug metabolism.

Methods Employed: Standard biochemical technics were employed.

Major Findings: 1) Distribution of components of mixed function oxidase between rough and smooth endoplasmic reticulum of liver cells. The microsomal fraction isolated from liver homogenates by differential centrifugation is known to consist of fragments of the endoplasmic reticulum pinched off during homogenization. In the intact hepatocyte, endoplasmic reticulum (ER) exists in two forms depending on the presence or absence of attached ribosomes: granular or rough-surfaced ER can be identified by the presence of ribosomes while agranular or smooth-surfaced ER lacks ribosomes. On homogenization, these two types of ER give rise to two kinds of structures, rough-surfaced and smooth-surfaced microsomes. Because of slight differences in specific gravity, rough and smooth microsomes can be separated in relatively high purity by discontinuous gradient centrifugation, and can be studied biochemically and morphologically.

Previous work, carried out by J. R. Fouts and co-workers, at the University of Iowa, demonstrated many hepatic microsomal NADPH-dependent enzymes to be highly concentrated in smooth microsomes. In the rabbit, there was 3 to 5 times as much activity in smooth microsomes as in rough for the metabolism of several substrates. The present experiments were carried out to determine the component of the microsomes which accounts for the differences in activity of the two fractions.

Male New Zealand rabbits (2-2.5 kg) and Sprague-Dawley rats (ca. 160 g) were used. Smooth and rough-surfaced microsomal subfractions were prepared by the method of Dallner. Mixed function oxidase activity when measured by the N-demethylation of ethylmorphine or the hydroxylation of aniline was found to be significantly higher in smooth endoplasmic reticulum than in rough. In the rabbit, the ratio of smooth to rough activities was significantly greater than 1 whether the activities were expressed per g liver (ratio of 5:1), per mg microsomal protein (ratio of 3-5:1), per μg phospholipid phosphorus (ratio of 2:1), per unit cytochrome P-450 (ratio of 1.7:1), or per unit NADPH cytochrome c reductase (ratio of 2:1). On the other hand, if the activities were

normalized to the NADPH-cytochrome P-450 reductase, there was no significant difference between the rough and smooth membranes. These results suggest that, in rabbit liver microsomes, the rate-limiting step of drug oxidation is the reduction of cytochrome P-450. By contrast, in the rat, the difference in activities can be explained by differences in the levels of cytochrome P-450 between smooth and rough membranes.

2) Role of various components of the drug oxidase system in species variation in hepatic microsomal drug metabolism. Species variation in hepatic microsomal drug metabolism has been long recognized and been shown to parallel differences in the duration of action of many drugs between animal species. As part of a general program in this laboratory, oriented toward elucidation of the rate-limiting step in microsomal oxidations, we examined several components of the microsomal oxidase system in several laboratory animal species. In accord with previous reports, we found considerable variation in ethylmorphine N-demethylase activity (Vmax) between four animal species (rat, mouse, guinea pig, rabbit). The data indicated that species variations in microsomal cytochrome P-450 content were not likely to account for the differences in demethylase activity since the former varied by only a factor of about 2 (0.67-1.23) while the latter varied by a factor of greater than 4 (4.2-18.0) between the species. Similarly, differences in the maximal spectral changes produced by addition of ethylmorphine to microsomal suspensions did not account for species differences in demethylase activity. Measurement of NADPH cytochrome c reductase activity, thought to be a component of the microsomal oxidase system, revealed only slight differences which again were inadequate to account for differences in demethylase activity. However, assays of microsomal NADPH cytochrome P-450 reductase activity in livers of several species revealed a rough proportionality with demethylase activity. Accordingly, expression of demethylase activity per unit cytochrome P-450 reductase activity yielded remarkably similar values except for sex differences.

These findings suggest that variations in hepatic microsomal ethylmorphine N-demethylase activity between animal species may be more closely related to variations in cytochrome P-450 reductase than to other components of the oxidase system heretofore examined. Although this explanation may account for interspecies differences in demethylase activity, it did not account for the sex difference in demethylase activity in rats or mice.

3) Effect of drug substrates on the reduction of hepatic microsomal cytochrome P-450 by NADPH. Previous work demonstrated that addition of various substrates and inhibitors to microsomal suspensions in the absence of NADPH caused two types of change in the difference spectrum: "type I" is characterized by a trough at about 420 mμ and a peak at about 385 mμ, whereas "type II" compounds produce a peak at about 430 mμ and a trough at about 394 mμ. Measurement of the rate of cytochrome P-450 reduction by NADPH revealed that "type I" compounds markedly stimulated the initial rate of reduction, while "type II" compounds significantly inhibited reduction. These effects were produced without significant changes in total reducible cytochrome P-450.

Cytochrome P-450 Reduction

Drug Substrates	Control	Presence of Substrate	conc. mM	% of control
Type I				
Ethylmorphine	16.2	28.7	0.4	177
Hexobarbital	11.0	18.7	0.6	170
SKF 525-A	12.4	27.4	0.1	221
Aminopyrine	12.2	17.3	0.5	142
Imipramine	11.7	19.1	0.5	162
Type II				
Aniline	15.3	6.9	1.5-2.0	45
Nicotinamide	11.5	6.8	10	59
DPEA	12.2	4.4	0.1	36

Other experiments revealed a slight but significant sex difference in the rate of cytochrome P-450 reduction by rat liver microsomes in the absence of drug substrate, but in the presence of ethylmorphine the rate of reduction was 69% greater in microsomes from males than from females. These findings suggested that the sex differences in Vmax for the metabolism of drug substrates may be related to the relative magnitude of substrate-enhanced rates of cytochrome P-450 reduction in the two sexes.

Significance to Biomedical Research and the Program of the Institute: These studies should provide a better understanding of the mechanisms involved in drug oxidation and the nature of the rate-limiting step therein.

Proposed Course of Project: We shall continue our studies of factors influencing cytochrome P-450 reduction in hepatic microsomes, expanding the scope of the investigation to other substrates and the effects of pretreatment with phenobarbital and 3-methylcholanthrene.

Honors and Awards: None.

Publications:

Gigon, Philippe L., Gram, Theodore E., and Gillette, James R.: Effect of drug substrates on the reduction of hepatic microsomal cytochrome P-450 by NADPH. Biochem. Biophys. Res. Commun., in press, 1968.

Davies, Donald S., Gigon, Philippe L. and Gillette, James R.: Sex differences in the kinetic constants for the N-demethylation of ethylmorphine by rat liver microsomes. Biochem. Pharmacol., in press.

504

Serial No.-NHI-189
1. Chemical Pharmacology
2. Enzymes Drug Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Interrelationship between TPNH-dependent nitroreductase and drug metabolizing enzyme systems in mouse liver microsomes

Previous Serial Number: NHI-320

Principal Investigator: Dr. James R. Gillette
Mr. Henry A. Sasame

Other Investigator: None

Cooperating Unit: None

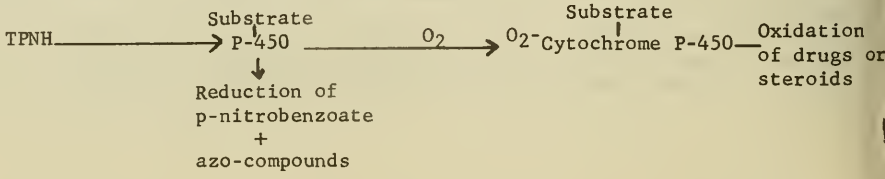
Man Years:

Total:	0.3
Professional:	0.3
Other:	None

Project Description:

Objectives: Liver microsomes contain TPNH-dependent enzymes which catalyze the reductions and the oxidations of drugs through a variety of reactions. The purpose of this project is to investigate the mechanism of nitroreductase and to uncover interrelationship between the oxidative and the reductive system.

Microsomes contain a cytochrome which in its reduced form combines with carbon monoxide to form a complex having an absorption maximum at 450 mμ. There is now considerable evidence that TPNH reduces this cytochrome P-450 - substrate complex and the reduced complex reacts with oxygen to form an "active oxygen" complex which transfers oxygen to various steroids and drugs. In the absence of a substrate, cytochrome P-450 is reduced and then oxygenated to form an "active oxygen" complex which decomposes to form hydrogen peroxide. There is also considerable evidence that the reduced cytochrome P-450 participates in the reduction of p-nitrobenzoate and certain azo compounds. These general reactions are illustrated below:



505

Methods Employed: Standard biochemical methods have been used. The determination of cytochrome P-450 was carried out by using the Shimadzu MPS 50 L Spectrophotometer.

Major Findings: 1) Effect of the amount of cytochrome P-450 on nitroreductase activity. Last year we reported that cytochrome P-450 was a component of the major nitroreductase in liver microsomes. This view was supported by facts that the reduction of p-nitrobenzoate was not only inhibited by carbon monoxide but also inhibited by compounds which cause a peak at 430 mμ in liver microsomes, but not by compounds which cause a trough at 420 mμ. Moreover, the inhibitory constant of an inhibitor to the nitroreductase activity in mouse liver microsomes agreed with the binding constants of the compound to the liver microsomes. In further support of this view, we now wish to report an almost stoichiometric relationship between the change in nitroreductase activity and the change in the amount of cytochrome P-450 in liver microsomes from either phenobarbital induced rats or CCl_4 treated mice. Accordingly, the elevation in nitroreductase activity due to phenobarbital induction was CO-sensitive. Furthermore, it is noteworthy that even though there was an almost 50% fall in both nitroreductase activity and the cytochrome P-450 in mouse liver microsomes from CCl_4 treated animals, there was no significant fall in TPNH cytochrome c reductase activity.

Since almost all oxidative drug-metabolizing enzyme activities are lost upon solubilization of microsomes, owing to the conversion of cytochrome P-450 to cytochrome P-420, one criterion of nitroreductase as a cytochrome P-450 mediated enzyme is its stability after solubilization. After treatment of mouse liver microsomes with 0.07% steapsin, the recovery of nitroreductase activity in the "solubilized" microsomes was about 20% or less. The finding of "NADPH-reducible cytochrome P-420" in the "solubilized" microsomes, however, may well account for the residual nitroreductase activity. It is noteworthy that the cytochrome P-420 is rapidly destroyed in aerobic incubation, under which all oxidative enzymes are assays, whereas nitroreductase is assayed anaerobically.

2) Activator. The subcellular distribution study of nitroreductase activity in mouse liver microsomes revealed the presence of an activator in all fractions. It was of interest that the sum of nitroreductase activity from boiled all other components in liver plus microsomal fraction can account for the activity in the whole homogenates. At first it was thought that the activator was a flavin. But acid treatment of microsomes, which is known to remove flavins, removed the activator. Moreover, the required amount of flavins (mM) to enhance nitroreductase activity was about three orders of magnitude higher than the total amount of flavin present in liver. In addition, the enhanced activity by flavins was partially CO-sensitive, whereas the stimulated activity by the activator in liver was completely CO-sensitive. The acidic acetone extract of liver microsomes, however, can account for 80% of the activation by the boiled microsomes. On the other hand, the phospholipide extract of liver microsomes accounted only for 40% of the activation by the boiled microsomes.

Significance to Biomedical Research and the Program of the Institute: These studies should provide a better understanding of the metabolism of drugs and other foreign compounds.

Proposed Course of Project: We shall continue our studies on the relationship between spectral changes and the mechanisms of oxidation and reduction by liver microsomal enzyme systems.

Honors and Awards: None.

Publications:

Sasame, Henry A.: Mechanism of inhibition of NADPH-dependent Enzymes in Liver Microsomes. Ph. D. Dissertation, George Washington Universit 1968.

Hernandez, Patrick H., Gillette, J. R. and Mazel, Paul: Studies on the mechanism of action of mammalian hepatic azoreductase--I. Azoreductas activity of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase. Biochem. Pharmacol. 16: 1859-1875, 1967.

Hernandez, Patrick H., Mazel, Paul and Gillette, J. R.: Studies on the mechanism of action of mammalian hepatic azoreductase--II. The effect of phenobarbital and 3-methylcholanthrene on carbon monoxide sensitive and insensitive azoreductase activities. Biochem. Pharmacol. 16: 1877-1888, 1967.

Serial No. -NHI-190
1. Chemical Pharmacology
2. Enzymes Drug Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Interaction of SKF-525A on liver microsomes, especially in regard to spectral changes

Previous Serial Number: None

Principal Investigators: Dr. James R. Gillette
Dr. Bitten Stripp

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.2
Professional:	0.2
Other:	0

Project Description:

Objectives: The addition of various substrates to liver microsomes causes spectral changes. Spectrophotometric studies suggest that these changes are related to substrate interaction with microsomal hemoprotein. The magnitude of these spectral changes is dependent on protein concentration, substrate concentration and substrate employed. The concentration of substrate necessary to produce half-maximal spectral changes (Ks) is similar to the concentration necessary to evoke half-maximal enzyme activity (Km). However, the extinction coefficient and the composition of the complexes have not been determined. Since SKF-525A is so highly bound to liver microsomes that its apparent Ks value varies with microsomal protein concentration, it seemed possible that values for the extinction coefficient and composition of the complex might be estimated by plotting the apparent Ks values against the protein concentration according to the following equation:

$$Ks' = Ks + 0.5\ (Bt/mg\ protein)\ [protein]$$

The intercept of the line is the true Ks and the slope is 0.5 the number of binding sites per mg protein. Once the number of binding sites is known the molar extinction coefficient is easily calculated.

Methods Employed: Standard biochemical techniques were employed. Difference spectra were recorded at a Shimadzu recording spectrophotometer and the amount of cytochrome P-450 was measured as the ΔOD at 450 mμ in microsomal samples treated with CO for 1 min, followed by the addition of a few mg of

505

$Na_2S_2O_4$. The experiments were performed with liver microsomes from rabbits, which have been induced for 3 days with phenobarbital (80 mg/kg) in order to get increased microsomal activity to make measurements possible with low protein concentrations.

Major Findings: By plotting the ΔOD against the amount of SKF-525A added (S) at protein concentrations ranging from 1 to 3 mg/ml in the microsomal suspension, curves were obtained with two different slopes. By means of the tangents to these slopes the concentration of SKF-525A necessary to produce half-maximal ΔOD was calculated for each protein concentration and the apparent dissociation constant Ks was plotted against protein concentration. There was a linear relationship, the intersection of the ordinate being 1.7×10^{-6} M, indicating the concentration of the SKF-525A in a protein-free solution. By means of this number and the Ks for 1 mg/ml solution, it could be calculated that 9.4 mμmoles of SKF-525A was bound to 1 mg of microsomal protein when maximal spectral change was observed. The molar extinction coefficient was 2450. At the same time the cytochrome P-450 content was measured to be 3.07 mμmoles/mg protein. Making the assumption that the SKF-525A bound to the microsomes under these conditions was associated solely with the cytochrome P-450, we calculated that 3 moles of SKF-525A is bound to 1 mole of P-450. The validity of this assumption, however, remains to be investigated.

Significance to Biomedical Research and the Program of the Institute: Since SKF-525A is a well-known potent inhibitor of drug metabolizing enzymes, the finding of the molar ratio between the inhibitor and one of the mixed function oxidase compounds (P-450) may provide a better understanding of the mechanism of inhibition of drug metabolism.

Proposed Course of Project: Since phenobarbital in rats increases the amount of cytochrome P-450 per mg microsomal protein, we shall compare the binding of SKF-525A to liver microsomes from control and phenobarbital pretreated rats. Similar studies will be carried out with liver microsomes from CCl_4 treated animals, in which cytochrome P-450 in liver microsomes is selectively destroyed. Other highly bound substrates and inhibitor will be similarly studied.

Honors and Awards: None.

Publications: None.

Serial No.- NHI-191
1. Chemical Pharmacology
2. Enzymes Drug Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of carbon tetrachloride on mixed function oxidase system in liver microsomes

Previous Serial Number: None

Principal Investigators: Dr. James R. Gillette
Dr. Jose A. Castro
Dr. Frank E. Greene
Mr. Henry A. Sasame
Dr. Bitten Stripp

Other Investigators: None

Cooperating Units: Dr. Castro receives a fellowship from the National Council of Scientific and Technical Investigators, Argentina

Dr. Greene receives a fellowship from the Division of Environmental Health Sciences

Man Years:

Total:	1.2
Professional:	1.1
Other:	0.1

Project Description:

Objectives: It is well known that carbon tetrachloride causes a marked impairment of drug-metabolizing enzymes of the liver. It has been suggested that the principal toxic action is produced by a metabolite rather than by carbon tetrachloride itself. The object of these experiments is to study the toxic effects of a fixed dose of carbon tetrachloride in a variety of experimental conditions known to influence the metabolism of a number of foreign compounds. Through these experiments we hope to determine if the toxic actions would be altered, and if the pattern of alteration observed will allow us to conclude that carbon tetrachloride either is, or is not metabolized to a more active species.

Methods Employed: Standard biochemical and analytical methods have been used.

Major Findings: The effect of CCl_4 on cytochrome P-450 and total heme:

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The impairment of oxidative enzymes by liver microsomes is accompanied by a decrease in cytochrome P-450. We have demonstrated that the destruction of cytochrome P-450 is not mediated through stimulation of lipid peroxidation, or by a solvent action of CCl_4. Other experiments have established that the decrease in cytochrome P-450 is accompanied by a decrease in the total heme content of liver microsomes. Moreover, the level of cytochrome b5, the other heme component of microsomes, was not changed.

An apparent association of the metabolism of CCl_4 to its destructive action on cytochrome P-450 was observed in newborn rats, an increasing effect on P-450 being seen with increasing age. At the same time, the metabolizing ability in the liver microsomes was increasing, as measured by ethylmorphine metabolism.

Additional support for this association was obtained in experiments in which carbon tetrachloride was given to animals pretreated with phenobarbital to induce microsomal enzymes. In these experiments in which the P-450 content and drug-metabolizing activity was tripled in controls, the destruction of P-450 by CCl_4 was increased from 39% to 76%.

In comparing the CCl_4 destruction of P-450 in male and female rats, no sex difference was seen. Although male rats generally show a higher metabolic activity for most substrates than female rats, there are substrates such as aniline and zoxazolamine which are metabolized by both sexes at about the same rate. Therefore the finding of an equal effect on cytochrome P-450 in male and female rats does not necessarily provide evidence against metabolic activation of CCl_4.

More difficult to reconcile with the activation theory of CCl_4 toxicity is our results with adrenalectomized rats. We had expected to find a decreased destruction of P-450 in adrenalectomized rats, since the activity of microsomal enzymes from such animals is lower than controls. However, the destruction of P-450 was actually higher in the adrenalectomized group.f

Inhibitions of drug metabolism and CCl_4: A number of compounds known to inhibit microsomal enzymes were tested for their ability to alter the action of CCl_4 on these enzymes. Of the compounds tested, only SKF-525A protected against the destruction of cytochrome P-450. Although this compound is the most potent inhibitor studied, the reason why other compounds, which are also potent inhibitors, showed no protective effect is not clear. However, even though SKF-525A did protect cytochrome P-450 from destruction, it did not prevent irreversible binding of the C^{14} to liver microsomal protein.

Significance to Biomedical Research and the Program of the Institute: These studies should provide a better understanding of the mechanisms of CCl_4-induced impairment of drug-metabolizing enzymes of liver microsomes. They also provide a means for altering the relative amounts of cytochrome P-450 and other components of the mixed function oxidase systems. Such alterations should be useful in elucidating the mechanism of these systems.

Although we have suggestive evidence that the toxic effects of CCl_4 on microsomal enzymes are mediated via a metabolite, no direct experimental evidence has been obtained to establish this relationship. We also do not know if CCl_4 is metabolized by a cytochrome P-450 enzyme, although there is some evidence to suggest that this is the case.

Experiments are planned which will study the formation of $C^{14}O_2$ from $C^{14}Cl_4$ in the presence of potent inhibitors of drug-metabolizing enzymes. Other studies will measure gas chromatographically the amount of $CHCl_3$ formed from CCl_4 by liver microsomes.

We also hope to use the carbon tetrachloride treated animal as a means of studying the "different types" of P-450 that have been reported.

Honors and Awards: None.

Publications:

Castro, J. A., Sasame, Henry A., Sussman, Howard and Gillette, James R.: Diverse effects of SKF-525A and antioxidants on carbon tetrachloride-induced changes in liver microsomal P-450 content and ethylmorphine metabolism. Life Sciences 7: 129-136, 1968.

Sasame, Henry A., Castro, Jose A. and Gillette, James R.: Studies on the destruction of liver microsomal cytochrome P-450 by carbon tetrachloride administration. Biochem. Pharmacol., in press.

512

Serial No. NHI-192
1. Chemical Pharmacology
2. Enzymes Drug Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The regulatory role of adrenal steroids on the mixed function oxygenase enzymes of liver microsomes

Previous Serial Number: None

Principal Investigators: Dr. J. A. Castro
Dr. F. E. Greene
Dr. P. Gigon
Mr. H. Sasame
Dr. J. R. Gillette

Other Investigators: None

Cooperating Units: Dr. Castro receives a fellowship from the National Council of Scientific and Technical Investigations, Argentina.

Dr. Gigon receives a fellowship from the Swiss Academy of Medical Science.

Dr. Greene is supported by a fellowship from the Division of Environmental Health Sciences.

Man Years:

Total:	0.6
Professional:	0.5
Other:	0.1

Project Description:

Objectives: The ability of the pituitary-adrenal system to influence the rate of metabolism of drugs by the mixed function oxygenase system of liver microsomes of male rats is well known. However, the responsiveness of the individual components of this system have not been studied.

The purpose of this project was to determine if specific points of regulation on this system could be detected by altering adrenal steroid levels in rats.

Methods Employed: Adrenalectomized and sham adrenalectomized Sprague-Dawley male rats were obtained from Hormone Assay Labs, Chicago, Illinois, and force-fed equivalent amounts of a liquid diet to reduce the possible "starvatio

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effects that accompany adrenalectomy. Certain groups of rats received an aqueous suspension of cortisone acetate, 5 mg/kg/day for 7 days prior to sacrifice. Standard biochemical techniques and assay procedures were used to estimate the activities or content of the specific components of the mixed function oxygenase system.

Major Findings: Adrenalectomy produces a decrease in overall activity of drug-metabolizing activity as measured by the demethylation of ethylmorphine.

The Km was increased and the Vmax decreased in adrenalectomized animals. However, the spectral dissociation constant (Ks) and maximal spectral change observed with ethylmorphine were not different from sham values.

The most striking changes seen following adrenalectomy were reduced activities of TPNH, cytochrome c reductase and P-450 reductase.

Cortisone administration restored the ability of microsomes to demethylate ethylmorphine. The activities of TPNH, cytochrome c reductase and P-450 reductase were increased above sham levels in the cortisone-treated adrenalectomized animals, while the P-450 content and special binding properties were not significantly changed, either by adrenalectomy or by cortisone administration.

These findings suggest that the steroids exert their influence by altering the rate of P-450 reduction, although it is not possible to state if either of the reductases that respond to adrenalectomy and cortisone is the principal site of action.

Significance to Biomedical Research and the Program of the Institute: These studies should provide insight into some endocrine factors that may alter rates of drug metabolism. They also provide a means for altering the relative amounts of the various components of the mixed function oxidase systems and thus should aid in elucidating the mechanisms of these enzyme systems.

Proposed Course of Project: The project has been completed and a manuscript is being prepared for publication.

Honors and Awards: None.

Publications:

Gillette, James R.: Individually different responses to drugs according to age, sex and functional or pathological state. In Ciba Foundation Symposium on Drug Responses in Man, London, J.& A. Churchill Ltd., 1967, pp. 24-49.

514

Serial No.-NHI-193
1. Chemical Pharmacology
2. Enzymes Drug Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The use of the regenerating liver as a tool to study mechanisms of NADPH-dependent ("drug-metabolizing") enzymes

Previous Serial Number: None

Principal Investigator: Dr. James R. Gillette

Others Investigators: Dr. T. E. Gram
Dr. A. M. Guarino
Dr. F. E. Greene
Dr. P. L. Gigon

Cooperating Units: Drs. Gram and Guarino are Research Associates in the Pharmacology-Toxicology Training Program, NIGMS.

Dr. Greene is a Special Fellow sponsored by the Division of Environmental Health Sciences, NIH.

Dr. Gigon is supported by the Swiss Academy of Medical Sciences.

Man Years:

Total:	2.2
Professional:	2.2
Other:	None.

Project Description:

Objectives: Despite the low levels of microsomal drug-metabolizing enzymes in fetal or newborn animal livers, the activities of these enzymes can be markedly stimulated by pretreating the animals with inducing agents such as phenobarbital (Pb), 3-methylcholanthrene (3-MC) or chlordane. Since the immature liver is relatively deficient in enzyme content but possesses the ability to respond to enzyme inducers, it was of interest to study the effects of inducers on livers which are rapidly proliferating following partial hepatectomy.

Methods Employed: Standard surgical and biochemical methods were employed

Major Findings: The results of the first phase of this investigation demonstrate that regenerating rat livers respond to injections of Pb or 3-MC with increases in the activity of p-nitroanisole demethylase, hexobarbital

55

hydroxylase and aniline hydroxylase. Injection of these inducers also caused increased microsomal content of cytochrome P-450. The extent to which P-450 content increases could be related to enzyme activity was similar for the substrates p-nitroanisole and aniline but not for hexobarbital. There appears to be a considerable advantage in using this tool, regenerating liver, over studies involving sex and strain differences in rates of drug metabolism. In studies including unoperated, sham operated and partially hepatectomized animals there is up to an 8-fold difference in the rate of metabolism between these different groups. This wide range of enzyme activity provides a more critical basis for studying some of the reported relationships between P-450 content and enzyme activity. Indeed, our results have shown that after Pb or 3-MC treatment of rats with regenerating livers, there is no consistent proportional relationship between enhanced enzyme activity and increased cytochrome P-450 content in both unoperated and operated animals.

Significance to Biomedical Research and the Program of the Institute: These studies should provide a better understanding of the mechanisms by which chronically administered drugs alter rates of drug metabolism.

Proposed Course of Project: This tool will be employed to test the effects of Pb on the drug-metabolizing constants Km and Vm as well as on the spectrally determined binding constants, Ks and Vs, for the substrate aniline.

Awards and Honors: None.

Publications:

Gram, T. E., Guarino, A. M., Greene, F. E., Gigon, P. L. and Gillette, J. R.: Effects of partial hepatectomy on the responsiveness of microsomal enzymes and cytochrome P-450 to phenobarbital or 3-methylcholanthrene. Biochem. Pharmacol.in press.

1. Methylation of the enzyme by AMe to form a methyl-B_{12} enzyme. This is considered the "priming" reaction.

2. Transfer of the methyl group to homocysteine to form methionine and a reduced B_{12} species on the enzyme.

3. Alkylation of the reduced B_{12} enzyme by N^5-methyl-H_4-folate.

4. Transfer of the methyl group to homocysteine and remethylation of the enzyme by N^5-methyl-H_4-folate.

The non-B_{12} enzyme has been partially purified and experiments are in progress to obtain an enzyme-substrate complex.

Role of GTP and Soluble Transfer Factors in Protein Synthesis

A requirement of GTP for the binding of F-met-tRNA to the ribosomes in the presence of initiation factors has been demonstrated. In addition two of the soluble factors required for polypeptide formation (Ts and Tu) bind GTP to form a Ts-Tu-GTP complex. Evidence has been obtained that this complex is involved in amino acyl-tRNA transfer to the ribosomes. The interactions of these protein factors with both GTP and amino acyl-tRNA is currently under investigation. In addition a third factor (G) has been shown to stimulate the reaction of phe-tRNA with puromycin in the presence of GTP. The data suggest that factor G is involved in the process of translocation. A binding of GTP to factor G has also been demonstrated. An *in vitro* protein synthesis system from rat brain has been developed and the effect of a number of biogenic amines and derivatives has been examined. Adrenochrome has been found to inhibit polypeptide formation markedly and its mode of inhibition is being investigated.

Synthesis of 2,3-Dihydroxybenzoyl Serine (DHBS)

This compound is produced in large quantities by *E. coli* when the iron level in the medium becomes limiting. The enzymatic synthesis involves the following reaction.

Serine + ATP + 2,3,-dihydroxybenzoic acid → DHBS

Two protein fractions are required for this conversion and the function of each protein is under investigation.

Cross-links of Proteins

A recently described congenital defect in fibrin cross-linking, the terminal step in blood clotting, is characterized by recurrent bleeding, and defective wound healing. We have identified the cross-link of normal polymerized fibrin. It is ε-(γ-glutamyl)lysine. This cross-link was absent in nonpolymerized fibrin prepared experimentally. It is highly probable that the explanation, at the molecular level, of the congenital defect of fibrin cross-linking is an inability to form ε-(γ-glutamyl)-lysine.

Kininogen-Kallikrein-Kinins System

Bradykinin and kallidin are peptides (kinins) which have several well-known potent actions in man. They produce pain, increase capillary permeability, mobilize leucocytes, contract isolated smooth muscle and are hypotensive. Kinins have been implicated in a number of physiological and pathological conditions and study of their biosynthesis, metabolism and pharmacology is crucial to an understanding of their importance in man. Kinins are formed by the action of specific enzymes (kallikreins) on a precursor protein, kininogen.

$$\text{Kininogen} \xrightarrow{\text{Kallikrein}} \text{Kinin}$$

Four kininogens have been isolated from human plasma and their relative significance is being assessed. They vary in structure, molecular weight, in their susceptibility to attack by the kallikreins and in their participation in the kinin-forming reaction observed when blood comes into contact with a foreign surface. One of the kininogens has proven to be an excellent antigen for the production of antibodies to bradykinin. This is a high-affinity antibody which, together with the C^{14}-bradykinin previously synthesized, has permitted the development of a very sensitive (2-60 ng) radioimmunoassay of bradykinin that is likely to supplant the cumbersome and unreliable bioassay.

Annual Report of the
LABORATORY OF CLINICAL BIOCHEMISTRY
National Heart Institute
July 1, 1967 through June 30, 1968

Amine Biogenesis and Metabolism

The relationship between sympathetic nerve activity and norepinephrine synthesis has been investigated further and direct evidence of a feed-back inhibition control of tyrosine hydroxylase has been obtained. Thus after administration of monoamine oxidase inhibitors, when tissue catecholamine levels rise, tyrosine hydroxylase activity decreases and less norepinephrine is formed. α-Adrenergic blocking agents, which are known to increase nerve activity, increase tyrosine hydroxylase activity markedly. This regulatory mechanism must be extremely important for the function of the sympathetic nervous system.

Interrelationships among the enzymes involved in norepinephrine metabolism are being investigated with the use of antibodies specific to each of the purified enzymes. Thus far antibodies to dopamine-β-hydroxylase and monoamine oxidase have been prepared. Fluorescent labeling of these antibodies has made it possible to detect the enzyme in cells.

Collagen Biosynthesis

Collagen proline hydroxylase has been purified at least 30-fold and to a state where many interfering enzymes are absent. With such preparations it has now been possible to show a stoichiometric oxidative decarboxylation of α-ketoglutarate accompanying the hydroxylation of proline residues in synthetic substrates. Neither pyruvate nor oxaloacetate can substitute for or spare α-ketoglutarate in this role. Oxidative decarboxylation of α-ketoglutarate presages a complicated electron transport system in the overall hydroxylation of proline residues. Such a system may require thiamine pyrophosphate and a ferredoxin-like protein.

Additional information has been obtained about the substrate requirements of the enzyme and it has been shown that proline residues in peptides, as small as hexapeptides, can be hydroxylated.

Aromatic Hydroxylation

Attempts have been made to detect epoxide intermediates during the hydroxylation of aromatic compounds. In the case of benzene-C^{14} it was shown that benzene epoxide is not a free intermediate in the hydroxylation to phenol. However, an enzyme-bound intermediate could not be ruled out. Studies with napthalene-epoxide are now underway.

Biosynthesis of Pteridines

Studies in bacteria on the biosynthesis of the pteridine cofactor for phenylalanine hydroxylation have revealed intermediates which are now being identified. A highly sensitive method for assay of the hydroxylation cofactor has been developed.

Actinomycin Synthesis

Phenoxazinone synthetase, an enzyme involved in the biosynthesis of actinomycin appears in the cells about 15 hours before the antibiotic is produced. The synthesis of the enzyme is under catabolic repression and the characteristics of the derepression of the enzyme have been studied. In addition good evidence has been obtained that 4-methyl-3-hydroxyanthranilic acid is an intermediate in the formation of actinomycin.

Synthesis of Methionine

Two enzymes have been described which catalyze methionine synthesis from homocysteine. One enzyme utilizes N^5-methyl-H_4-folate (monoglutarate) as the methyl donor and requires S-adenosylmethionine (AMe), a cobamide prosthetic group and a reducing system. The other reaction uses the triglutamate derivative of N^5-methyl-H_4-folate and only requires Mg as a cofactor.

Recent data have shown that the cobamide participates directly in methyl transfer from N^5-methyl-H_4-folate to homocysteine. Both AMe and the folate substrate are able to methylate the enzyme bound cobamide to form a methyl-B_{12} enzyme. The methylated enzyme quantitatively transfers its methyl group to homocysteine to form methionine. The sequence of reactions appear to be as follows:

Human plasma kallikrein and two urinary kallikreins are being purified and characterized chemically and enzymologically. They differ in their actions on the kininogens reacting at different rates and forming either bradykinin or kallidin.

Peptide catabolites of C^{14}-bradykinin infused into man have been detected in urine. Extension of this work to the identification of the peptides and their determination in urine could open new and important opportunities for the study of kinins in man in normal and pathological states.

Identification, Biosynthesis and Metabolism of Physiologically Active Peptides

The bradykinin containing peptide isolated from the venom of wasps has been synthesized by cooperating chemists in Germany. It will be compared pharmacologically and chemically to the natural peptide in order to prove the proposed structure.

Synthesis of the hypotensive and smooth muscle contracting peptide isolated from the skin of Rana pipiens has been attempted by the Merrifield solid phase method and by conventional means. The Merrifield method has been disappointing but the product obtained by conventional techniques is biologically active. An explanation is currently being sought for its low potency relative to the natural peptide.

Biomedical Application of Gas Chromatography

As an outgrowth of our need to determine the amino acid sequence of minute quantities of peptides, a gas chromatographic technique for the analysis of the phenylthiohydantoin derivatives of amino acids has been developed. Application of the technique to the structural determination of the peptide hormone, thyrocalcitonin has shown that this technique offers superior sensitivity, speed, resolving power, and ease of quantitation. Wide acceptance of the technique awaits further application now in progress.

Serial No.- NHI-194
1. Lab. Clinical Biochemistry
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NIH

Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Biosynthesis of Norepinephrine

Previous Serial Number: NHI-145

Principal Investigator: Sidney Udenfriend

Other Investigators: Wallace Dairman
Robert Gordon
Boyd Hartman
Olga Nikodijevic (Guest Worker), supported by The Upjohn Co.
Deltcho K. Zhelyaskov (Guest Worker on Riker International Fellowship in Pharmacology)

Cooperating Units: Sydney Spector and Albert Sjoerdsma, NHI, Experimental Therapeutics Branch; Morris Lipton, University of North Carolina, Chapel Hill, N.C.

Man Years:
Total: 4.5
Professional: 3.8
Other: .7

Project Description:

Work has continued on the production of specific antibody to dopamine-β-hydroxylase. Further investigations, using the sensitive methods of immunoelectrophoresis and disc-gel electrophoresis, have shown that the antibody produced previously was not sufficiently pure for fluorescent localization of the enzyme at the cellular level. This antibody is, therefore, being

utilized in the development of a radioimmune assay for the enzyme. The radioimmune assay will permit the quantitation of the enzyme in crude tissue systems. Such quantitation has not been possible before, because, standard assay techniques depending on enzyme activity, are unreliable due to the presence of native enzyme inhibitors.

Dopamine-β-hydroxylase has now been purified three fold greater than before. The enzyme in this highly pure form was then chromatographed on arcylamide gel. The band representing the enzyme (visualized using a fluorescent technique developed in this laboratory) was cut from the gel, and has been used for immunization of rabbits. It is hoped that the new antibody will be of sufficient specificity for cellular localization using fluorescent labeling techniques.

Investigation of norepinephrine control and synthesis using immunochemical methods: At the present time there is no adequate method for accurately quantitating the amounts of dopamine-β-hydroxylase in tissue. Methods which use enzyme activity give variable results because of the presence of native inhibitors. It is important to investigate the absolute levels of this and other enzymes, and to correlate the enzyme levels with the levels of norepinephrine found in various areas of the nervous system. Such comparisons will lead to a better understanding of basic control mechanisms.

Toward this end a radioimmune assay is being developed for dopamine-β-hydroxylase which will allow the quantitation of the enzyme even in the inhibited state. Also using fluorescent antibody techniques it may be possible to localize the enzyme at the cellular level. This information can then be correlated with other histochemical data about the distribution of norepinephrine in nervous tissue.

Regulation of catecholamine biosynthesis in vivo: The in vivo incorporation of label from a tracer dose of tyrosine-^{14}C into the heart norepinephrine of hypothyroid rats was determined and found to be two fold higher than controls. The rate of catecholamine synthesis in the brains and adrenals was not elevated with respect to control rats. Endogenous levels of catecholamines were similar for both groups of animals when

expressed on a per gram basis for heart and brain and on a pair bases for adrenals. However, in the plasma and tissue, the radiospecific activity of the precursor tyrosine was found to be elevated approximately 70% in the hypothyroid rats. It would appear that there is a significant increase in the rate of norepinephrine synthesis in the heart of the hypothyroid rat.

A study was undertaken to evaluate the effects of α-blocking agents on the rate of catecholamine synthesis in vivo. It was found that phentolamine and phenoxybenzamine increase the formation of radioactive norepinephrine and epinephrine in rat tissues when L-tyrosine-^{14}C is used as a precursor but not when L-dopa-^{3}H is the precursor. These findings indicate that α-blocking agents increase catecholamine synthesis by stimulating tyrosine hydroxylase activity.

Isolation and purification of synaptic vesicles from heart and brain. Currently, studies are being carried out to see if catecholamine storage vesicles can be isolated, purified and administered to rabbits to form antibodies. Attempts will be made to see if the formation of catecholamine storage granules can be preincubated in new-born animals thus decreasing organ catecholamine content.

Honors and Awards: Dr. Sidney Udenfriend received the Gairdner Foundation Award, Nov. 1967.

Publications:

1) Lipton, M.A., Gordon, R., Guroff, G., and Udenfriend, S.: p-Chlorophenylalanine induced chemical manifestations of phenylketonuria in rats. Science 156: 248-250, April 1967.

2) Engelman, K., Jequier, E., Udenfriend, S., and Sjoerdsma, A.: Metabolism of α-methyltyrosine in man: Relationship to its potency as an inhibitor of catecholamine biosynthesis. Journal of Clinical Investigation 47: 568-576, March 1968.

3) Spector, S., Gordon, R., Sjoerdsma, A., and Udenfriend, S.: End-product inhibition of tyrosine hydroxylase as a possible mechanism for regulation of norepinephrine

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part B: continued

synthesis. Molecular Pharmacology 3: 549-555, Nov. 1967.

Serial No.-NHI-195
1. Lab. of Clinical Biochemistry
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Hydroxyproline and Collagen

Previous Serial Number: NHI-146

Principal Investigators: John P. Comstock
T. John Gribble
Arnold Marglin
Sidney Udenfriend

Other Investigators: Robert E. Rhoads

Cooperating Units: Bernhard Witkop, National Institute of Arthritis and Metabolic Diseases, Laboratory of Chemistry. Dr. J. Kurtz and Dr. A. Berger, The Weizmann Institute of Science, Rehovoth, Israel

Man Years:
Total: 4-3/4
Professional: 3-3/4
Other: 1

Project Description:

The investigation of collagen biosynthesis has been extended to fibroblasts in tissue culture. Earlier studies of Howard Green in 1964, which used hydroxyproline formation as a measure of collagen, indicated that collagen was synthesized mainly in the stationary growth phase of fibroblast cultures. The present study is a further investigation of collagen biosynthesis during the growth of fibroblasts. The recently developed tritium release assay of Hutton et al. permits the direct measurement of collagen proline hydroxylase and its substrate, the unhydroxylated polypeptide chain. The enzyme activity was found to be

low in the rapidly dividing cell, and to rise rapidly as the growth rate slowed. Hydroxyproline synthesis correlated very well with the enzyme activity.

Substrate synthesis was found not to be coincident with the enzyme activity, but rather to parallel that of general cell proteins. The lack of coincidence between the synthesis of the polypeptide chain and the enzyme activity raises interesting questions about the dynamics of collagen biosynthesis which are now being investigated.

Recently it has been possible to produce within 3-4 hours a several fold increase in enzyme activity by artifically concentrating the cells at the proper time in the growth curve. Experiments are now in progress to determine if the increased collagen proline hydroxylase activity represents new protein synthesis or activation of a pre-existing protein.

The exact site of hydroxylation has still not been determined. Hydroxylation may be coincidental with protein synthesis, resulting in the growing peptide chain being hydroxylated on the ribosome, or a completed protocollagen molecule may be released from the ribosome, then hydroxylated. Our data are compatable with hydroxylation occurring with protein synthesis. It has been possible to isolate by gel filtration material of molecular weight less than 100,000 which contains hydroxyproline. Separate experiments have shown that a ribosomal system from chick embryoes can incorporate proline into a protein that functions as a substrate for collagen proline hydroxylase. This material migrated with the ribosomal peak during sucrose density gradients and could be released for the ribosome to a low molecular weight material by sodium dodecyl sulfate. It remains to be shown that this material is bound to the ribosome by amino acyl linkage and that this linkage persists during the hydroxylation reaction.

1. In attempt to purify collagen proline hydroxylase from fetal rat skin large losses in enzyme activity were encountered. It was found that the addition of serum albumin to incubation mixtures could restore activity to apparently inactive enzyme preparations, stimulating hydroxyproline formation by as much as fifteen-fold. Further investigation of this phenomenon did

not lead to an explanation of the effect. However, two facts indicated that it might be related to thiol groups: prior oxidation of the albumin abolished its stimulatory effect; also, certain thiol compounds were found to stimulate hydroxylation in a similar way but to a somewhat lesser extent. The enzyme could now be more highly purified.

2. Work was continued in the area of collagen-like synthetic polymers as substrates for collagen proline hydroxylase. In order to confirm the results obtained with the randomly polymerized polymer, H-(glycyl prolyl proline)$_n$-OH, a series of peptides of exactly known composition and molecular weight was synthesized by the solid-state method. It was shown that a peptide as small as H(glycyl prolyl proline)$_2$-OH could be hydroxylated by the enzyme. Kinetic data on peptides smaller than twenty-four amino acid residues could not be obtained because of considerable substrate inhibition. At present a thirty-six amino acid peptide with sequence corresponding to a recently elucidated portion of the collagen molecule is being synthesized.

3. Work on synthetic peptides was severly hampered by the fact that the time course of the enzyme was linear for only twenty minutes. In an attempt to improve the time course the enzyme catalase was tested and found effective, extending the period of linearily to 80 minutes. Under such conditions the extent of reaction was increased substantially and to such a degree that the disappearance of the cofactor α-ketoglutarate, previously shown to occur at low levels, was now large and easily measured. The disappearance of α-ketoglutarate was shown to occur simultaneously with and to require the same cofactors as the hydroxylation of proline.

Honors and Awards: None

Publications:

1) Rhoads, R., Hutton, J.J., and Udenfriend, S.: Factors which stimulate collagen proline hydroxylase. Archives of Biochem. Biophys. 122: 805-807, Dec. 1967.

Serial No. -NHI-195
1. Lab. of Clinical Biochem.
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NIH

Individual Project Report
July 1, 1967 through June 30, 1968

Part B: continued

2) Hutton, J.J., Kaplan, A., and Udenfriend, S.: Conversion of the amino acid sequence gly-pro-pro in protein to gly-pro-hyp by collagen proline hydroxylase. Archives of Biochem. Biophys. 121: 384-391, Aug. 1967.

3) Mussini, E., Hutton, J.J., and Udenfriend, S.: Collagen proline hydroxylase activity during wound healing, granuloma formation, scurvy and growth. Science 157: 927-929, Aug. 1967.

Serial No. -NHI-196
1. Lab. of Clinical Biochem.
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Metabolism of Aromatic Amino Acids

Previous Serial Number: NHI-159

Principal Investigator: Gordon Guroff

Other Investigators: Sidney Udenfriend
Jean Renson
Perola Zaltzman-Nirenberg

Cooperating Units: John Daly, Don Jerina, Bernhard Witkop, Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases

Man Years:

Total:	0.7
Professional:	0.7
Other:	0

Project Description:

Observations on the mechanism of the phenylalanine hydroxylase reaction have led to detailed studies on the action of many aromatic hydroxylase enzymes. A large number of these hydroxylase reactions involve hydroxylation-induced migrations of groups attached to the aromatic ring.

The phenylalanine hydroxylase reaction can thus be written in the form:

p-X-phenylalanine $\longrightarrow$ m-X-tyrosine

-where X is deuterium, tritium, chlorine, bromine, or methyl. Quantitative studies on the migration of tritium indicate that a charged intermediate is formed during the hydroxylation. Using preparations of liver microsomes, the nature of the intermediates leading to tritium migration in a variety of substrates have been described.

The hydroxylation of p-methylphenylalanine with phenylalanine hydroxylase gave rise not only to methyl migration but also to methyl hydroxylation. This observation shows the versatility of aromatic hydroxylase enzymes and also provides the first example of a side-chain hydroxylation requiring the reduced pteridine cofactor.

These studies have now yielded substantial information. Among other things the information obtained has a) provided new data on the mechanism of hydroxylase enzymes, b) suggested new criteria for judging nonenzymatic model systems and c) allowed the development of enzymatic assay tools not available in the past.

Honors and Awards: None

Publications:

1. Guroff, G., Daly, J., Renson, J., Jerina, D., Witkop, B., and Udenfriend, S.: Hydroxylation-induced migration: The NIH Shift. Science 157: 1524-1530, Sept. 1967.

2. Guroff, G., and Daly, J.: Quantitative studies on the hydroxylation-induced migration of deuterium and tritium during phenylalanine hydroxylation. Arch. Biochem. Biophys. 122: 212-217, Oct., 1967

3. Daly, J., Guroff, G., Udenfriend, S. and Witkop, B.: Hydroxylation-induced migrations of tritium in several substrates of liver aryl hydroxylases. Arch. Biochem. and Biophys. 122: 218-223, Oct., 1967.

4. Daly, J., Guroff, G., Udenfriend, S., and Witkop, B.: Hydroxylation of alkyl and halogen substituted anilines and acetanilides by microsomal hydroxylases. Biochem. Pharmac. 17: 31-36, Jan., 1968

5. Jerina, D., Guroff, G., and Daly, J.: Enzymatic and non-enzymatic hydroxylation and chlorination of p-deuteroanisole. Arch. Biochem. Biophys. 124: 612-615, Mar. 1968.

Serial No. -NHI-197
1. Lab. of Clinical Biochem.
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Pteridine Metabolism and Function

Previous Serial Number: NHI-152

Principal Investigator: Gordon Guroff

Other Investigators: Carol Rhoads
Ammon Abramowitz

Cooperating Units: None

Man Years:
Total: 1.5
Professional: 0.5
Other: 1.0

Project Description:

The biosynthesis of pteridines in Pseudomonas sp. is being studies. The overall biosynthetic pathway has been determined and attempts are being made to separate the various enzymes involved. Information on the mechanism of these various steps should allow the designing of inhibitors which may be of therapeutic interest.

A sensitive method for the quantitative determination of reduced pteridines has been developed. This method is based on the ability of reduced pteridines to serve as cofactors for the hydroxylation of phenylalanine by phenylalanine hydroxylase. Use of this method should facilitate studies of physiological and pharmacological influences on cofactor levels in various animal tissues.

Although it is known that reduced pteridines serve as cofactors in several important hydroxylations e.g., phenylalanine

hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, the exact structure of the pteridine in various biological sites is unknown. Only in rat liver has the exact structure been worked out. Accordingly, studies are under way which aim to determine the exact structure of the pteridine cofactors from various biological sites.

Honors and Awards: None

Publications:

1. Guroff, G., Rhoads, C.A., and Abramowitz, A.: A simple radioisotope method for phenylalanine hydroxylase cofactor. Anal. Biochem. 21: 273-278, Nov. 1967.

Serial No. -NHI-198
1. Lab. of Clinical Biochem.
2. Enzymes and Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Actinomycin Biosynthesis

Previous Serial No.: NHI-147

Principal Investigator: Vida Beaven

Other Investigators: Herbert Weissbach
Richard Marshall

Cooperating Unit: Edward Katz, Georgetown University School of Medicine and Dentistry, Washington, D.C.

Man Years:

Total:	1.2
Professional:	1.2
Other:	0

Project Description:

Studies have continued on the biosynthesis of actinomycin. In order to understand the factors which initiate antibiotic synthesis by Streptomyces antibioticus, the appearance of the enzyme phenoxazinone synthetase, which catalyzes the formation of actinocin, the antibiotic chromophore, has been examined in the growing cultures. De novo synthesis of this enzyme occurs between 9 and 36 hours after inoculation. Before this time the synthesis of the enzyme appears to be under catabolic repression. The rise in phenoxazinone synthetase is specific; a number of other enzyme activities were shown to remain unchanged or to decrease during these hours. Concomitant with the initiation of synthesis of phenoxazinone synthetase, the organism shows a decreased ability to take up actinomycin and becomes resistant to the antibiotic it will subsequently produce.

Earlier studies in this laboratory have provided evidence that 4-methyl-3-hydroxyanthranilic acid (MHA) is an intermediate in the conversion of tryptophan to actinomycin by washed cells of S. antibioticus. In vitro experiments were continued to determine at what point methylation of the aromatic ring occurs. When a cell-free preparation of S. antibioticus is incubated with 3-hhydroxyanthranilic acid, ^{14}C-methyl-S-adenosylmethionine, ATP, and mercaptoethanol, an acid extractable methyl labeled product is formed. This enzymatic reaction is dependent on time, enzyme concentration, 3-hydroxyanthranilic acid, and mercaptoethanol. The product does not appear to be MHA but rather a related methylated derivative.

Current studies involve an examination for possible intracellular precursors of the actinomycin molecule.

Honors and Awards: None

Publications:

1. Marshall, R., Redfield, B., Katz, E., and Weissbach, H.: Changes in phenoxazinone synthetase activity during the growth cycle of Streptomyces antibioticus. Arch. Biochem. Biophys. 123: 317-323, Feb. 1968.

2. Perlman, D., Mauger, A.B., and Weissbach, H.: Microbial transformation of peptide antibiotics. I. Degradation of actinomycins by Actinoplanes species. Antimicrobial Agents and Chemotherapy, 581-586, 1966.

3. Yoshida, T., Mauger, A., Weissbach, H., and Katz, E.: Actinomycin biosynthesis. Effect of structural and stereochemical methyl-proline isomers. J. Bacteriol. 95: 952-958, March 1968.

Serial No.-NHI-199
1. Lab. of Clinical Biochem.
2. Enzymes and Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Cobamide-Dependent Synthesis of Methionine

Previous Serial No. NHI-149

Principal Investigator: Robert T. Taylor

Other Investigators: Carolyn Whitfield
Robert J. Ertel
Herbert Weissbach

Cooperating Units: None

Man Years:

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

A specific cobamide containing protein that is present in extracts of Escherichia coli catalyzes the synthesis of methionine according to Reaction 1:

$$N^5\text{-Methyl-}H_4\text{-folate} + \text{Homocysteine} \xrightarrow[\text{S-Adenosyl-L-methionine (AMe)}\\ \text{Reducing system}]{B_{12}\text{-Enzyme}} \text{Methionine} + H_4\text{-Folate}$$

In this conversion both the reducing agent (generally reduced flavin) and AMe are required only in catalytic quantities; hence, the methyl group of methionine is derived almost exclusively from N^5-methyl-H_4-folate. An attractive hypothesis

has been that Reaction 1 proceeds via two steps, the first consisting of the transfer of a methyl group to a reduced cobalt on the enzyme to form a methyl-B_{12}-enzyme. In the second step the methyl group would be transferred off the cobalt to homocysteine yielding methionine and presumably regenerating the reduced B_{12}-enzyme.

Conclusive evidence has been obtained that the methyl groups of both AMe and N^5-methyl-H_4-folate can give rise to a methyl-B_{12}-enzyme. This was demonstrated by incubating substrate amounts of purified enzyme with methyl-^{14}C-H_4-folate (plus unlabeled AMe) in the presence of a $FMNH_2$-dithiothreitol reducing system. Methyl-^{14}C-B_{12}-enzyme formation in these experiments was proven upon the subsequent hot alcohol extraction of free methyl-^{14}C-B_{12} from the protein. Yields of methyl-^{14}C-B_{12}-enzyme were 1.0 and 0.5 to 0.6 equivalents per equivalent of bound B_{12} for methyl-^{14}C-AMe and N^5-methyl-^{14}C-H_4-folate, respectively, and methyl-^{14}C-B_{12}-enzyme formation from N^5-methyl-^{14}C-H_4-folate required exogenous, unlabeled AMe. On the other hand, unlabeled N^5-methyl-H_4-folate decreased methyl-^{14}C-B_{12}-enzyme formation from methyl-^{14}C-AMe to about 0.1 equivalent per equivalent of bound-B_{12} when both methyl group donors were present at nearly equal levels. A methyl-B_{12}-enzyme is also formed when methyl iodide is substituted as the methylating agent. Provided care is taken not to denature the protein a methyl-^{14}C-B_{12}-enzyme is active catalytically and will transfer 80-90% of its radioactivity to homocysteine aerobically in the absence of exogenous AMe.

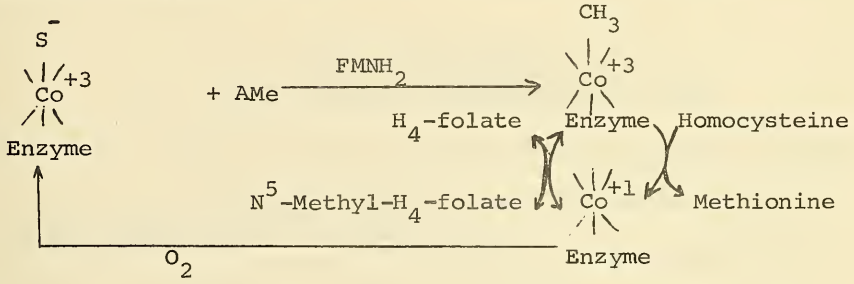
More recent experiments have dealt primarily with the reaction sequence by which the methyl-^{14}C group of N^5-methyl-^{14}C-H_4-folate becomes incorporated into a methyl-^{14}C-B_{12}-enzyme and the function of AMe in this process. Time studies have shown that within the first 15 seconds of incubation the B_{12}-enzyme is first methylated by AMe and that over the subsequent 2 minutes most of the methyl groups derived from AMe are replaced by those from N^5-methyl-H_4-folate. This conclusion was reached by incubating mμmole amounts of B_{12}-enzyme with methyl-^{14}C-AMe + N^5-methyl-H_4-folate and conversely with unlabeled AMe + N^5-methyl-^{14}C-H_4-folate. It was confirmed by determining the time course of radioactive methyl-B_{12}-enzyme formation when incubation mixtures contained methyl-(^{3}H)-AMe + N^5-methyl-^{14}C-H_4-folate.

It was further observed that a methyl-^{14}C-B_{12}-enzyme which has been isolated by Sephadex gel filtration loses its methyl-^{14}C group when it is incubated aerobically with either H_4-folate or unlabeled N^5-methyl-H_4-folate. Free N^5-methyl-^{14}C-H_4-folate was formed in these reactions.

The unpublished results in the preceeding paragraph indicated that, unlike the initial B_{12}-enzyme, a methyl-B_{12} enzyme might catalyze limited methionine synthesis in the absence of exogenous AMe and a reducing system. Indeed this was found to be so. Vitamin-B_{12}-enzyme first incubated with either AMe or methyl iodide under conditions which yield a methyl-B_{12}-enzyme formed 8-10 enzyme equivalents of methyl-^{14}C-methionine when incubated aerobically with N^5-methyl-^{14}C-H_4-folate plus homocysteine. This enzymatic turnover was 85% complete within 15 seconds and the resulting enzyme was no longer activated. In a N_2 atmosphere or in an evacuated system the amount of turnover was increased to 120-fold. Exogenous AMe had no significant effect either aerobically or anaerobically on this limited synthesis of methionine by an isolated methyl-B_{12}-enzyme. Prior incubation of the methylated (activated) enzyme with homocysteine destroyed its ability to catalyze limited methionine formation.

During the course of this past year the inhibition of chemical propylation (i.e. formation of a propyl-B_{12}-enzyme with propyl iodide) by AMe and N^5-methyl-H_4-folate was examined in more detail as was the identity of the cobamide that is extractable with hot ethanol directly off the B_{12}-enzyme as it is isolated. The ability of AMe to prevent chemical propylation was correlated directly with its ability to methylate the B_{12} chromophore on the enzyme; yet, only 1-2 enzyme equivalents of homocysteine can completely reverse this inhibition by demethylating the methyl-B_{12}-enzyme. Over 400 enzyme equivalents of homocysteine are necessary to effect a reversal of the inhibition of propylation by N^5-methyl-H_4-folate. All of the data gathered thus far indicate that the B_{12} chromophore on the enzyme (as it is isolated) extracts off the denatured protein in hot alcohol as sulfito-B_{12} and this suggests that the cobalt may be liganded to a sulfur atom prior to reduction and methylation.

A scheme to account for most of the observed enzymatic and chemical properties of the purified B_{12}-enzyme is summarized below (Reaction 2):



According to this reaction mechanism AMe would function in its normal manner as a methyl group donor but only methylate the initial molecules of enzyme and the small amounts of enzyme which became reoxidized during the course of the reaction in a continuous flavin reducing system. Consequently, activation by AMe would be accomplished in a catalytic manner. Reaction sequence 2 also accomodates the fact that this enzyme catalyzes a very slow transmethylation reaction between methyl-^{14}C-AMe, itself, and homocysteine even in the presence of excess unlabeled N^5-methyl-H_4-folate, the preferred methyl group donor. Reaction sequence 2 furthermore explains the ability of methyl iodide to substitute for AMe as a cofactor in the catalysis of Reaction 1. Replacement of the methyl group of AMe by the methyl-^{14}C group of N^5-methyl-^{14}C-H_4-folate (in the absence of homocysteine) may simply involve a direct methyl group exchange reaction between a methyl-B_{12}-enzyme and N^5-methyl-^{14}C-H_4-folate. Current experiments are being designed to clarify this point and to test further the mechanism suggested in Reaction 2.

Part B: Yes

Serial No.-NHI-199
1. Lab. of Clinical Biochem.
2. Enzymes and Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part B:

Honors and Awards: Dr. Herbert Weissbach received the Dept. Superior Service Award - DHEW

Publications:

1. Dickerman, H., Taylor, R.T., and Weissbach, H.: Unusual growth characteristics of a methionine-cyano-B_{12} auxotroph of Escherichia coli. J. Bact. 94: 1609-1615, Nov. 1967.

2. Taylor, R.T., and Weissbach, H.: Isolation of methyl-B_{12} from Escherichia coli B N^5-methyl-H_4-folate-homocysteine vitamin-B_{12} transmethylase. Biochem. Biophys. Res. Commun. 27: 398-403, July 1967.

3. Taylor, R.T., and Weissbach, H.: Escherichia coli B N^5-methyltetrahydrofolate-homocysteine vitamin-B_{12} transmethylase: Formation and photolability of a methylcobalamin enzyme. Arch. Biochem. Biophys. 123: 109-126, Jan. 1968.

4. Taylor, R.T., Whitfield, C., and Weissbach, H: Chemical propylation of vitamin-B_{12} transmethylase: Anomalous behavior of S-adenosyl-L-methionine. Arch. Biochem. Biophys. 125: 240-252, April 1968.

Serial No.-NHI-200
1. Lab. of Clinical Biochem.
2. Enzymes and Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Role of GTP and Soluble Transfer Factors in Protein Synthesis

Previous Serial Number: NHI-150

Principal Investigators: Herbert Weissbach
Robert Ertel
Nathan Brot

Other Investigator: Betty Redfield

Cooperating Units: Dr. Jorge Allende - Laboratory of Biochemical Genetics, NHI

Man Years:

Total:	3.4
Professional:	2.4
Other:	1.0

Project Description:

In previous studies the enzyme, N^{10}-formyl-H_4-folate-methionyl-tRNA transformylase was purified from extracts of E. coli. In a continuation of studies on the formation and function of formyl-methionine-tRNA the requirements for the binding of formyl-methionine-tRNA to ribosome in the presence of the triplet AUG and initiation factors (2 proteins isolated from the ribosome by washing with 1 M NH_4Cl) were investigated further. At 0.004 M Mg an absolute GTP dependency was demonstrated for the binding of formyl-methionine-tRNA to the ribosomes. In addition a protein component in the initiation factor preparation complexed ^{3}H-GTP; the complex was assayed by

retention on a millipore filter or after chromatography with Sephadex G-25. Using the millipore technique as a routine means to assay the formation of the GTP complex the two proteins have been purified from the supernatant fraction of E. coli B extracts which are both needed to form the GTP complex. These two proteins correspond to the soluble transfer factors Ts and Tu which are required for polymerization. The third transfer factor (G) needed for polymerization is not involved in the formation of the Ts-Tu-GTP complex. Tu and Ts are also required, in the presence of GTP, for enzymatic binding of amino acyl-tRNA (AA-tRANS) to the ribosomes. These results in addition to studies reported by Ravel et al. and Gordon suggest the following reaction sequence:

$$Ts + Tu + GTP \rightarrow Ts\text{-}Tu\text{-}GTP$$

$$Ts\text{-}Tu\text{-}GTP + AA\text{-}tRNA \rightarrow Ts\text{-}Tu\text{-}GTP\text{-}AA\text{-}tRNA$$

$$Ts\text{-}Tu\text{-}GTP\text{-}AA\text{-}tRNA + Ribosomes \xrightarrow{Messenger} Ribosome\text{-}Messenger\text{-}AA\text{-}tRNA$$

Studies are continuing on the purification of Ts and Tu with the hope of obtaining a better understanding of the interactions of GTP and AA-tRNA with these protein components.

In addition, the role of Factor G in the polymerization reaction has been under investigation. This protein also catalyzes a hydrolysis of GTP in the presence of ribosomes, and has been postulated to function in the process of translocation. The antibiotic puromycin has been used to investigate this problem, since it has been well established that puromycin reacts as an analogue of AA-tRNA. The reaction of AA-tRNA, bound to ribosomes, with puromycin to yield puromycin peptides is known to be stimulated by soluble factors. The rationale for the observed stimulation is that the soluble factors are required for the movement (translocation) of the AA-tRNA from a site on the ribosome which is unreactive towards puromycin to a more reactive site. Purified fractions of G have now been shown to replace the soluble factor, in the presence of GTP. In addition a binding of GTP to Factor G, in the presence of ribosomes has been demonstrated.

Part B: Yes

Serial No. NHI-200
1. Lab. of Clinical Biochem.
2. Enzymes and Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part B:

Honors and Awards: None

Publications:

1. Caskey, C.T., Redfield, B., and Weissbach, H.: Formylation of guinea-pig liver methionyl-sRNA. Arch. Biochem. Biophys. 120: 119-123, Apr. 1967.

2. Dickerman, H.W. and Weissbach, H.: N^{10}-formyl tetrahydrofolate: Methionyl tRNA(F) transformylase from Escherichia coli B. Methods in Enzymology 12B Grossman, L. and Moldave, K. (Eds.), 1968.

3. Weissbach, H., and Redfield, B.: Deformylation of N-formylmethionine by E. coli extracts. Biochem. Biophys. Res. Commun. 27: 7-11, Apr. 1967.

4. Allende, J.E. and Weissbach, H.: GTP interaction with a protein synthesis initiation factor preparation from Escherichia coli. Biochem. Biophys. Res. Commun. 28: 82-88, July 1967.

5. Allende, J.E., Seeds, N.W., Conway, T.W. and Weissbach, H.: Guanosine triphosphate interaction with amino acid polymerization factor from E. coli. Proc. Natl. Acad. Sci. 58: 1566-1573, Oct. 1967.

6. Ertel, T., Brot, N., Redfield, B., Allende, J.E. and Weissbach, H.: Binding of GTP by soluble factors required for polypeptide synthesis. Proc. Natl. Acad. Sci. 59: 861-868, Mar. 1968.

Serial No. -NHI-201
1. Lab. of Clinical Biochem.
2. Enzymes and Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on 2,3-Dihydroxybenzoylserine, a New Metabolic Product from Escherichia coli

Previous Serial Number: NHI-153

Principal Investigator: Nathan Brot

Other Investigator: Richard Weller

Cooperating Unit: J. Goodwin, National Institute of Allergy and Infectious Diseases

Man Years:
Total: 1.5
Professional: 0.5
Other: 1.0

Project Description:

Earlier studies have shown that 2,3-dihydroxybenzoylserine (DHBS) fails to accumulate in the growth medium of E. coli which has been supplemented with 2.0×10^{-6}M $FeSO_4$. In addition, under these conditions, the synthesis of the enzyme which catalyzes the terminal step in the synthesis of DHBS from 2,3-dihydroxybenzoic acid and serine is repressed.

In an attempt to ascertain whether one of the functions of DHBS was to act as a chelator of iron and transport it into the cell, DHBS was chelated with ^{59}Fe and then incubated with both growing and stationary phase cells. It was shown that iron in this form could be incorporated into the cell, but that there was no difference in the rate of uptake of the chelated iron when compared with controls in which only free iron was added. Thus it would appear that the function of DHBS is not to

facilitate the incorporation of iron into the cell. It is possible, however, that the iron bound to the DHBS is selectively incorporated into a specific cellular molecule as has been found to be the case with ferrichrome bound iron.

It has been found that under optimum conditions, as much as 100 mg/liter of DHBS accumulates in the growth medium. Thus it was of interest to see if, under these conditions, there were any changes in the activities of the last three enzymes which ultimately convert 3-phosphoglyceric acid to serine. It was found that the specific activities of these enzymes were constant regardless of whether or not DHBS was produced. Thus the normal synthesis of serine in these cells appears to be sufficient for both the production of DHBS and for other metabolic requirements.

DHBS has recently been chemically synthesized and attempts will be made to determine, in a variety of systems, whether it possesses any biological activity. Experiments are currently in progress to purify the DHBS synthetase enzyme and study its mechanism of action.

Honors and Awards: None

Publications:

1. Brot, N., and Goodwin, J.: Regulation of 2,3-dihydroxybenzoylserine synthetase by iron. J. Biol. Chem. 243: 510-513, Feb. 1968.

Serial No. -NHI-202
1. Lab. of Clinical Biochem.
2. Enzymes and Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on N^5-methyltetrahydropteroyl-triglutamate(methyl-THF-G_3)-homocysteine transmethylase in E. coli

Previous Serial Number: None

Principal Investigator: Carolyn Whitfield

Other Investigator: Herbert Weissbach

Cooperating Units: None

Man Years:

Total:	1.2
Professional:	0.2
Other:	1.0

Project Description:

In the early 1960's D.D. Woods described two enzymatic reactions leading to the formation of methionine from homocysteine in E. coli. One of these reactions is catalyzed by a cobamide containing enzyme, which is only present in cells grown on vitamin B_{12}. In the absence of the vitamin the non-B_{12} enzyme, methyl-THF-G_3-homocysteine transmethylase, functions.[12] During the past year, work on the non-B_{12} enzyme was initiated in an attempt to compare the mechanism of the non-B_{12} with the B_{12} dependent reaction. The non-B_{12} enzyme catalyzes the following reaction: methyl-THF-G_3 + homocysteine $\xrightarrow{Mg^{++}}$ THF-G_3 + methionine. A radioactive assay has been devised utilizing C^{14}-methyl-THF-G_3 which was chemically synthesized from C^{14}-formaldehyde and THF-G_3 by a slight modification of the procedure published by Donaldson and Keresztesy. C^{14}-methyl-THF-G_3

transfers its methyl group to homocysteine enzymatically to give C^{14}-methionine, which can be separated from the substrate using a Dowex-1 column. The assay has the expected dependencies and is linear with respect to time and enzyme concentration. The purification of this enzyme is now underway. A procedure has been developed thus far to obtain an enzyme 30 or more fold purified. In addition, the enzyme levels in the crude extract have been derepressed three fold on a small scale by growing a methionine requiring mutant on D-methionine. Attempts are now being made to reproduce this on a large scale. Preliminary studies on the control of this enzyme show that it is also repressed by methionine and by vitamin B_{12}.

During the next year, the non-B_{12} enzyme will be purified on a larger scale in order to investigate its mechanism of action by the following methods: 1) studies on the formation of an enzyme-substrate complex, 2) studies on the role of Mg^{++}, 3) studies on the substrate specificity, 4) studies on the thiol activation, and 5) kinetic studies. The control of the enzyme will also be investigated.

Honors and Awards: None

Publications: None

Serial No.-NHI-203
1. Lab. of Clinical Biochem.
2. Enzymes and Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Brain Protein Synthesis and Nucleic Acid Function

Previous Serial Number: None

Principal Investigator: Frederick Goodwin

Other Investigator: Herbert Weissbach

Cooperating Units: None

Man Years:

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

A. A system has been developed to study brain protein synthesis in vitro. Using C^{14}-phenylalanyl-tRNA, prepared from E. coli, and ribosomes and a soluble enzyme fraction prepared from rat brain, excellent incorporation of C^{14}-phenylalanine into hot TCA insoluble material is obtained with NH_4Cl washed ribosomes. The polymerization reaction shows absolutely dependencies on soluble enzymes, synthetic messenger (poly U) and ribosomes. The role of the transferase enzymes will be examined more closely and initial attempts at purification of these factors have begun. The effect of various amines and psychoactive drugs on the polymerization reaction will be investigated.

B. In conjunction with the above studies, the binding of biogenic amines and psychoactive drugs to macromolecules has been examined using either Sephadex G-25 chromatography or

millipore filtration as a means of assay. It has been possible to show that several biogenic amines (including norepinephrine, dopamine and serotonin) bind to brain ribosomes. The nature of the binding and the possible effects of these compounds on brain synthesis of macromolecules are being studied.

Honors and Awards: None

Publications: None

Serial No.-NHI-204
1. Lab. of Clinical Biochem.
2. Sec. of Peptide Biochem.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Unusual Peptide Linkages in Proteins

Previous Serial Number: NHI-156

Principal Investigator: John J. Pisano

Other Investigators: Marjorie Peyton

Cooperating Units: John Finlayson, Division of Biological Standards, Laboratory of Blood and Blood Products

Man Years:

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

Using a method for the enzymic digestion of proteins, we have been able to isolate β-aspartylglycine, γ-glutamylglycine and ε-(γ-glutamyl)lysine in digests of fibrin. Convincing evidence has been obtained to show that ε-(γ-glutamyl)lysine bonds are formed during the cross-linking of fibrin by the action of FSF, the cross-linking enzyme. Approximately 2 moles of this bond were found per mole of cross-linked fibrin. Insignificant amounts were found in soluble fibrin and intermediate amounts in fibrin in which the cross-linking was inhibited by glycine ethyl ester (GEE). GEE apparently acts as a competitive inhibitor of cross-linking. This theory was supported by the finding of large quantities of γ-glutamylglycine, in fibrin in which the cross-linking was inhibited by GEE. The peptide was not found in cross-linked fibrin. A study in which glycine-1-C^{14} ethyl ester was used as inhibitor led to the same conclusion. Glysine C^{14} was incorporated into both the γ-glutamylglycine and β-aspartylglycine recovered. The presence of β-aspartyl-

glycine and γ-glutamylglycine bonds in these digests has been shown to have no relation to cross-linking.

A procedure for determination of cross-linked lysine in proteins based on cyanoethylation has given results for fibrin which are in excellent agreement with those obtained by enzymic digestion followed by analysis for ε-(γ-glutamyl)lysine. Insoluble, soluble and GEE-inhibited clots were found to have blocked lysines in amounts equivalent to the ε-(γ-glutamyl)-lysine found after enzymic hydrolysis. Currently we are attempting to develop suitable methods for solubilizing and cyanoethylating collagen, amyloid and other cross-linked proteins in order to determine the possible involvement of lysine.

Honors and Awards: None

Publications: None

Serial No.-NHI-205
1. Lab. of Clinical Biochem.
2. Sec. of Peptide Biochem.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Kallikrein-Kininogen-Kinin System

Previous Serial Number: NHI-193, 194

Principal Investigators: Marion E. Webster
S. Jacobsen
J.V. Pierce

Other Investigators: S.I. Said
William Anderson, Jr. (Technician)

Cooperating Units: Laboratory of Chemical Pharmacology, National Heart Institute and the Medical College of Virginia, Richmond, Virginia

Man Years:

Total:	1.8
Professional:	.9
Other:	.9

Project Description:

Objectives; Methods employed: Investigations were continued on the isolation and purification of the various components of this enzyme system. Previous studies (Pierce and Webster in Hypotensive Peptides, 1966) have resulted in the isolation from human plasma of two kininogens both with a molecular weight around 50,000. Recently Jacobsen and Kriz (Br. J. Pharmac. Chemother. 29: 25, 1967) have isolated a third substrate with molecular weight around 200,000. It appeared possible that this substrate represented component B as proposed by Margolis and Bishop (Aust. J. Exptl. Biol. Med. Sci. 41: 239, 1963) and that glass treatment of human plasma would cause its exhaustion. In order to test this hypothesis, plasma was chromatographed on DEAE-Sephadex.

Major findings: These studies have confirmed the presence of two classes of kininogens in human plasma (50,000 and 200,000 molecular weight) and that human plasma kallikrein can form kinins in a quick reaction only from the latter substrate. In addition, they have demonstrated that still another type of kininogen is present in human plasma from which only trypsin can form kinins. It was initially thought that this new kininogen represented degradative products of the other two classes of kininogens. However, it has a molecular weight greater than 30,000, does not exhibit cross-reactions with antisera to the 50,000 molecular weight kininogens, competes directly with bradykinin in the radioimmunoassay and its kinin content is unaffected by carboxypeptidase B. The kinin moiety in these kininogen(s) may be located at least in part at the N-terminal portion of the molecule. The other two classes of kininogens exhibit immunological cross-reactions, compete directly with bradykinin in the radioimmunoassay and their kinin content is partially destroyed by pretreatment with carboxypeptidase B indicating that each is composed of two forms of kininogens - one with the kinin moiety located at the C-terminal portion of the molecule. As other evidence suggests that the remaining portion of these kininogens contain the kinin sequence within the polypeptide chain, it would appear unlikely that the new kininogen(s) are derived from the former.

Glass activation of normal human plasma destroys the kinin content of only the 200,000 molecular weight kininogen regardless of the enzyme used to form kinins i.e. trypsin, human urinary kallikrein or human plasma kallikrein. Hageman deficient plasma, on the other hand, does not appreciably alter the ability of the various enzymes to form kinins from these kininogens. These data would support the hypothesis that component B is identical with the 200,000 molecular weight substrate and that glass treatment of human plasma activates endogenous human plasma kallikrein by a process dependent on Hageman factor. The alternate explanation that the glass particles had so altered the residual kininogen that it could no longer form kinins with human plasma kallikrein, is no longer possible. Acid treatment of plasma, on the other hand, has no immediate effect on the three classes of kininogens, but following neutralization and incubation for two hours at 37° trypsin can still form kinins from the large molecular weight substrate, but human urinary kallikrein and human plasma kallikrein are unaffective suggesting that endogneous

human plasma kallikrein was activated by this treatment, but was capable of forming kinin from only a portion of this substrate. No evidence for the presence of two kallikreins as proposed by Vogt (Arch. Exptl. Pathol. Pharmakol. 256: 127, 1967) could be found in these studies.

Studies on the radioimmunoassay are now complete. With the availability of (2,3-L-proline-^{14}C) bradykinin triacetate the procedure is now as sensitive as the rat bioassay and quantities of bradykinin from 2-64 ng/ml can now be quantitated. When compared with the bioassay method, this procedure has advantages in specificity, accuracy, simplicity and rapidty, and, as labeled bradykinin with a higher specific activity is synthesized, has the potential for increased sensitivity. Methods for the preparation of high-titer and high-affinity antibody to derivatives of bradykinin still remain a major problem.

Earlier studies had provided evidence to suggest that conversion of prekallikrein to kallikrein was the result of a series of pre-enzyme to enzyme conversions - each enzyme in succession activating the next until prekallikrein is converted to kallikrein. Preliminary kinetic studies would suggest that they are activated in the following order - enzyme IV, III, V, II and I. In this series, enzyme IV is identified as Hageman factor and enzymes II and I as PF/dil and human plasma kallikrein respectively.

In collaboration with Dr. S.I. Said, attempts were made to identify one or more of the hypotensive substances in lung extracts as a kinin, kallikrein or kallikrein activator. No kinin could be detected in the extracts or in the partially purified fractions. Addition of these materials to dog plasma *in vitro* did not result in the formation of detectable kinins. However, since high concentrations of kininase are present and might prevent the detection of kinin formation *in vitro*, further experiments are planned to determine whether kininogen levels are lowered or kinin levels elevated following intravenous injection of these hypotensive substances.

Proposed course of project - To continue studies on various aspects of the kallikrein-kininogen-kinin system.

Honors and Awards: None

Publications:

1. Webster, M.E. and Pierce, J.V.: Studies on the enzymes involved in the activation of human plasma kallikrein. In Rocha e Silva, M. and Rothschild, H.A. (Eds.) International Symposium on Vasoactive Polypeptides: Bradykinin and Related Kinins. III Int. Pharm. Congr. and Soc. Bras. Farm. Ter. Exp., Sao Paulo, 155-160, Aug. 1967.

2. Skinner, N.S., Jr., and Webster, M.E.: Submaxillary gland blood flow: The role of kinins and beta-adrenergic receptors. Fed. Proc. 27: 76-79, 1968.

Serial No.-NHI-206
1. Lab. of Clinical Biochem.
2. Sec. of Peptide Biochem.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Synthesis of Radioactive Peptides of Biomedical Interest

Previous Serial Number: None

Principal Investigators: S. Udenfriend
John J. Pisano

Other Investigators: Evelyn Attix - Administrative Officer, Intramural Research, NHI

Cooperating Units: Dr. K. Frank Austen, Harvard Med. School
Dr. F. Merlin Bumpus, Cleveland Clinic
Dr. Ervin G. Erdos, Univ. of Oklahoma Med. Center
New England Nuclear Corp., Boston, Mass.

Man Years: Total: 0.1; Professional: 0.1; Other: 0

Project Description:

As an outgrowth of our research needs, our laboratory has initiated contracts for the synthesis of radioactive peptides of bio-medical interest by private companies. This was undertaken because it was believed other laboratories would have similar needs but could not afford the high cost. An advisory committee consisting of international authorities from universities and a research institute gave the plan its enthusiastic support. Through the auspices of the Special Research Projects Branch of the Office of the Associate Director for Extramural Programs, a contract was written.

After careful deliberation by the Committee, the New England Nuclear Corporation was awarded the contract because of the unique nature of the project and because they agreed to

accept the guidance provided by us and our consultant Dr. Bruce Merrifield, of the Rockefeller University. The company has fulfilled all contracts. At a total cost of $48,475, we have received 20 μmoles of 2-proline-C^{14}-bradykinin having a specific activity of 29 μc/mg; 1.03 g of nonlabeled bradykinin and 25 μmoles 2,3 proline-C^{14}-bradykinin having a specific activity of 200 μc/μmole.

Preliminary studies with the radioactive peptides by selected researchers has been gratifying. For example, we have infused the labeled peptide in man and detected urinary metabolites. This could open up many avenues of research relating to the kinins in normal and pathological states. Drs. Austen, of Harvard, and Drs. Webster and Pierce, of our Institute, have developed superior radioimmunoassays. Dr. John Stewart of the Rockefeller University finds the labeled peptide is invaluable for studying the mechanism of pulmonary inactivation of bradykinin.

Having established the quality and utility of the synthetic peptide, we have drafted an advertisement to be published in Science and other suitable journals which will notify the scientific community that qualified researchers can obtain from us 20 μc of the C^{14}-bradykinin and 20 mg of the nonlabeled peptide.

Currently under consideration are 4 competitive bids from private companies to synthesize.

A. Nonlabeled Peptides	Quantity
1. Kallidin	1.0
2. Methionylkallidin	0.5 g
3. (1-aspartic-5 isoleucine) angiotensin II	1.0 g

B. Radioactive Peptides	Min. S.A.	
1. (3,4,Proline-C^{14} U.L.) Kallidin	200 μc/μmole	20 μmoles
2. (1-Aspartic-5 isoleucine C^{14} U.L.) Angiotensin II	200 μc/μmole	20 μmoles

If this program is as successful as the first, other peptides of bio-medical interests will be synthesized.

Part B: None

Serial No.-NHI-207
1. Lab. of Clinical Biochem.
2. Sec. of Peptide Biochem.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Metabolism of Bradykinin in Man

Previous Serial Number: None

Principal Investigator: John J. Pisano

Other Investigators: Marjorie Peyton

Cooperating Units: Harry Keiser and Albert Sjoerdsma, Experimental Therapeutics Branch, NHI

Man Years:

Total:	0.4
Professional:	0.4
Other:	0

Project Description:

Radioactive bradykinin has been synthesized through a special contract initiated by this laboratory and supported through the Special Research Projects Branch of the NHI. This ^{14}C peptide is the first intrinsically labeled bradykinin ever prepared and is invaluable for metabolic studies. It has been infused into man, and urinary metabolites have been detected. Studies, to date, in two individuals show similar excretion patterns. One radioactive metabolite has been isolated and shown to be a peptide fragment of bradykinin. Its exact structure is currently under study. The peptide will be infused into other individuals to establish the metabolic profile. Once characteristic metabolites of bradykinin are established, analys will be undertaken of urines from patients with a variety of diseases thought to involve kinins. Malignant carcinoid will be studied first because patients with this disease are suspected of having a high turnover of bradykinin.

Part B: None

Serial No.- NHI-208
1. Lab. of Clinical Biochem.
2. Sec. of Peptide Biochem.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Preparation of Antibodies to Bradykinin

Previous Serial Number: None

Principal Investigator: Jack V. Pierce

Other Investigators: None

Cooperating Units: M.E. Webster, Laboratory of Clinical Biochemistry, Mr. Leonard D. Stuart, NIH Animal Center, LA

Man Years:

Total:	0.3
Professional:	0.3
Other:	0

Project Description:

Progress during the past year: Attempts to repeat previous experiments in which disc gel bands of kininogen I elicited antibodies to bradykinin in sheep have so far failed, although the titer of precipitating antibodies to the kininogens was as high as that found in antiserums containing high-affinity antibodies to bradykinin. There are at least two possible explanations. First, the sheep in the three successful experiments were Suffolk cross-bred, whereas the unsuccessful immunizations were done on Hampshire sheep. Second, in the latter experiments, the kininogen I disc gel sections or the kininogen I solution used to make fresh disc gels had been stored for at least a year at -20°.

Direction of current research: Kininogen I newly prepared from a mixture of kininogens I and II by hydroxylapatite chromatography will be electrophoresed on disc gels, and the excised

kininogen I bands will be injected into both a Suffolk cross-bred and a Hampshire sheep.

Attempts will be made to couple bradykinin to bromoacetyl-cellulose and to use the product for adsorbing anti-bradykinin from sheep antiserums to kininogen I.

Honors and Awards: None

Publications: None

Serial No.-NHI-209
1. Lab. of Clinical Biochem.
2. Sec. of Peptide Biochem.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Purification of Human Urinary and Plasma Kallikreins

Previous Serial Number: None

Principal Investigator: Jack V. Pierce

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 0.2
Professional: 0.2
Other: 0

Project Description:

Progress during the past year: Hydroxylapatite chromatography of crude human urinary kallikrein with a phosphate gradient in the presence of high concentrations of NaCl or KCl gave two approximately equal activity peaks, each with about 15-fold purification. In the absence of salt, no such separation and little purification were achieved. Electrofocusing of peak I gave two TAMe peaks, one at pH 4.2 and the other at pH 4.4, in a ratio of 2:1. The removal of sialic acid groups, known to be labile at pH 4.0 and below, may account for these variants.

Electrofocusing of a human plasma kallikrein preparation (about 1% pure) gave three peaks of TAMe activity, the major one being associated with a copious precipitate which formed at pH 5.0-5.4 in the gradient. This experiment is being repeated on material from which the euglobulin has been removed by precipitation at pH 5.2 in the absence of salt.

Direction of current research: These kallikreins will be further purified to obtain suitable material for preparing antiserums. It is hoped that immunoadsorbents made from them can be used to remove both prekallikrein and kallikrein directly from human plasma, the former with the purpose of illuminating the mechanism whereby it is activated. The purification of human urinary kallikrein is being pursued both for its own sake and for the possibility that it has at least one antigenic site in common with plasma kallikrein and the kallikreins from other tissues, in case the purification of the plasma enzyme proves to be too difficult. An immunoadsorbent requires only only combining site on the antigen, whereas antigen-antibody precipitation requires at least two combining sites.

Work will be done on determining the nature of the active enzyme site of these kallikreins, e.g., by reacting them with such trypsin-specific reagents as TLCK and ethyl p-guanidinobenzoate.

Honors and Awards: None

Publications: None

Serial No.-NHI-210
1. Lab. of Clinical Biochem.
2. Sec. of Peptide Biochem.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Isolation and Characterization of Human Plasma Kininogens

Previous Serial Number: None

Principal Investigator: Jack V. Pierce

Other Investigators: None

Cooperating Units: M.E. Webster, Laboratory of Clinical Biochemistry, NHI

Man Years:

Total:	0.5
Professional:	0.5
Other:	0

Project Description:

Progress during the past year: Disc gel electrophoresis of a mixture of kininogens I and II, plus some impurities, after treatment with human plasma kallikrein gave results consistent with the models proposed for them ("Hypotensive Peptides," Springer-Verlag, 1966, p. 130; Federation Proc. 27, 52, 1968). Human plasma kallikrein-treated kininogen I gave a band with a higher mobility toward the anode than untreated I, but a lower mobility than that of the band obtained with human urinary kallikrein. Plasma kallikrein-treated kininogen II gave a band with a lower mobility than untreated II, whereas with human urinary kallikrein there is no mobility change. These differences can be ascribed to the net charge change caused by the removal of bradykinin by human plasma kallikrein and of kallidin (lysyl-bradykinin) by urinary kallikrein. Thus, the presence of active kininogens I and II can be detected qualitatively even in fairly crude preparations.

562

Direction of current research: We plan to obtain more pure kininogen II from human plasma, react it with a kallikrein, and isolate enough of the small, probably carboxyl-terminal fragment to study some of its properties and to prepare antibody to it or to couple it to a suitable support to remove any antibodies which may be present in sheep antiserums to kininogen II. The isolated antibodies will in turn be coupled to a support to adsorb kininogen II directly from plasma. The sam approach with kininogen I and anti-bradykinin will be tried. For this purpose, we shall repeat the immunization of sheep with disc gel bands of kininogen I to obtain more antibody to bradykinin. The purpose of the immunological technique is to provide a relatively simple means of kininogen assay and isolation. High-affinity antibody to bradykinin would also be a great value in clinical studies.

We plan also to purify and characterize the 200,000 molecular weight kininogens of human plasma which were first described by S. Jacobsen.

Honors and Awards: None

Publications:

1. Pierce, J.V.: Structural Features of Plasma Kinins and Kininogens. Federation Proc. 27: 52-57, Jan-Feb. 1968.

Serial No.-NHI-211
1. Lab. of Clinical Biochem.
2. Sec. of Peptide Biochem.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Role of the Kinins in Inflammation

Previous Serial Number: None

Principal Investigators: Marion E. Webster
Harriet Maling

Other Investigators: Mrs. Martha A. Williams
Mr. William Anderson, Jr.

Cooperating Units: Dr. Harriet Maling and Mrs. Martha A. Williams are members of the Laboratory of Chemical Pharmacology, National Heart Institute

Man Years:
Total: 0.2
Professional: 0.1
Other: 0.1

Project Description:

Objectives: It has been proposed that the kinins may contribute to the inflammatory synovial reaction seen in arthritides of varying etiology. Earlier investigations (Melmon, Webster, Goldfinger and Seegmiller, Arthritis Rheumat. 10, 13, 1967) have shown that synovial fluid from these patients contain kinins and that an acute attack in two gouty patients (spontaneous or induced by microcrystalline sodium urate) resulted in increased kinin levels 24 hrs after the onset of symptoms. However, for a substance to be implicated in the etiology of a disease, it should be demonstrated that blocking or inactivation of the substance will cause relief of symptoms. Unfortunately, no compounds capable of suppressing kinin action are available that can be used in patients. In dogs Phelps, Proko and McCarty (J. Lab.

5.64

Clin. Med. 68, 433, 1966) reported that carboxypeptidase B suppressed the inflammatory response to injected bradykinin but did not alter the synovitis induced by microcrystalline urate crystals. However, it is a well known peculiarity of dog plasma that it does not form kinin when brought into contact with glass. It is possible, therefore, that, unlike humans, the kinins are not liberated in crystal-induced synovitis in this species. The purpose of the present investigation is to delineate the contribution of the kinins to the inflammatory reaction seen in the paws of rats following the injection of sodium urate crystals. The rat was selected as the animal of choice since the formation of kinin by endogenous plasma kallikrein is similar to that of humans.

Methods employed: Inflammation was produced in the rat's paw by bradykinin, sodium urate crystals and other inflammatory agents. An equal volume of diluent was injected into the other paw and the degree of inflammation determined by the weight of the paws after amputation.

Major findings: Preliminary studies have shown that uric acid crystals are capable of inducing inflammation in the paws of rats. As in the human, this is not an immediate response. Inflammation slowly progresses to a maximum in 6 hours and this inflammation is maintained for a period of at least 18 hours. In contrast, the inflammation exhibited by cellulose sulphate or carrageenin reaches a maximum in 3 to 4 hours and then declines. The inflammation exhibited by all three agents, however, is partially inhibited by systemic administration of soy bean trypsin inhibitor while that given by bradykinin was not. These data suggest that a protease is involved in the production of this edema and are consistant with the theory that the soy bean trypsin inhibitor is inhibiting PF/dil, an activator of plasma kallikrein, or plasma kallikrein and thus preventing the formation of kinins.

Proposed course of project: To continue investigation of other substances such as carboxypeptidase B, ellagic acid etc. in an effort to determine the contribution of the kinins to this inflammation.

Part B: Yes

Honors and Awards: None

Publications:

1. Webster, M.E.: Human plasma kallikrein, its activation and pathological role. Fed. Proc. 27: 84-89, Jan-Feb., 1968.

Serial No. -NHI-212
1. Lab. of Clinical Biochemistry
2. Sec. of Peptide Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Identification, Biosynthesis and Metabolism of Physiologically Active Peptides

Previous Serial Number: NHI-157

Principal Investigator: John J. Pisano

Other Investigator: Takenori Tanimura

Cooperating Units: Terumi Nakajima, Tokyo University

Man Years:

Total:	1.3
Professional:	1.3
Other:	0

Project Description:

Synthetic Polistes kinin (see NHI-127) has just been prepared by Dr. E. Schroder. It will be compared to the natural peptide (we isolated from wasp venom) using chemical and pharmacological tests so that we can verify our proposed structure. Other pharmacological studies are also contemplated in view of its relationship to bradykinin, its potent actions and its unique natural source. A new peptide from the extracts of the skin of Rana pipiens which has both hypotensive and smooth muscle contracting activity was isolated and the structure elucidated as indicated in last years report.

1 2 3 4 5 6 7 8 9 10 11
Pyr-Val-Pro-Glu-Try-Val-Ala-Gly-His-Phe-Met-NH_2

Synthesis of this peptide has been attempted by both Merrifield's solid phase method and conventional techniques. The compound synthesized by conventional means is biologically active, but that obtained by the solid phase method has not been tested due to the presence of many containing peptides which have been difficult to remove. The yields by the solid phase method have also been very low as determined by amino acid analysis.

The method of synthesis of the peptide is:

1	2	3	4	5	6	7	8	9	10	11
Pyr	Val	Pro	Glu	Try	Val	Ala	Gly	His	Phe	Met

Z-Ala-Gly-OEt
Z-Val - Ala-Gly-OEt
Z-Try - Val - Ala-Gly-OEt
Z-Glu - Try - Val - Ala-Gly-OEt
Z-Pro - Glu - Try - Val - Ala-Gly-OEt — t-Boc-Phe-Met-OCH_3
Z-Val - Pro - Glu - Try - Val - Ala-Gly-OEt — Z-His-$NHNH_2$ — Phe-Met-OCH_3
Z-Val - Pro - Glu - Try - Val - Ala-Gly-OH — Z-His — Phe-Met-OCH_3
Pyr-TCP Val - Pro - Glu - Try - Val - Ala-Gly-OH — Z-His — Phe-Met-NH_2
Pyr-Val - Pro - Glu - Try - Val - Ala-Gly-OH — His — Phe-Met-NH_2
Pyr-Val - Pro - Glu - Try - Val - Ala-Gly — His — Phe-Met-NH_2

Comparison of the Chemical and pharmacological properties of the synthesized and natural peptides are under study.

Dr. Terumi Nakajima, our former associate now at the University of Tokyo, has isolated a new peptide from the skin of a Japanese frog closely related to our Rana pipiens.

1	2	3	4	5	6	7	8	9
Val	Pro	Pro	Gly	Phe	Thr	Pro	Phe	Arg

It is a bradykinin analogue in which the N-terminal Arg is replaced by Val and Ser at position-6 is replaced by Thr. This is the first bradykinin analogue isolated from a natural source. It is unusual in that it does not contain the basic N-terminal arginine which has long been considered essential for bradykinin activity. A similar peptide may be present in man.

In the presence of this possibility, we have synthesized the peptide by the following method.

Synthesis of Val^1-Thr^6-bradykinin

1	2	3	4	5	6	7	8	9
Val	Pro	Pro	Gly	Phe	Thr	Pro	Phe	Arg

Z-Pro-Pro-OCH_3 — Z-Phe-Thr-OCH_3 — Z-Phe-NO_2-Arg-OCH_3
Z-Pro-Pro-OH — H-Gly-OEt — Z-Phe-Thr-$NHNH_2$ — Z-Pro - Phe-NO_2-Arg-OCH_3
Z-Pro-Pro — Gly-OEt — Z-Phe-Thr — Pro - Phe-NO_2-Arg-OCH_3
Z-Val - Pro-Pro — Gly-OEt
Z-Val - Pro-Pro — Gly OH — Phe-Thr — Pro - Phe-NO_2-Arg-OCH_3
Z-Val - Pro-Pro — Gly — Phe-Thr — Pro - Phe-NO_2-Arg-OCH_3
H-Val - Pro-Pro — Gly — Phe-Thr — Pro - Phe — Arg-OCH_3
H-Val - Pro-Pro — Gly — Phe-Thr — Pro - Phe — Arg-OH

Chemical and pharmacological comparisons of the synthetic and natural peptides are in progress.

Honors and Awards: None

Publications:

1. Pisano, J.J.: Studies on the Structure of the Major Kinin in Wasp Venom. Inter. Symposium on Vaso-Active Polypeptides: Bradykinin and Related Kinins, Sao Paulo, Brazil, August, 1966.

Serial No.-NHI-213
1. Lab. of Clinical Biochem.
2. Sec. of Peptide Biochem.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Application of Gas Chromatography to Problems in Biochemistry and Medicine

Previous Serial Number: NHI-161

Principal Investigator: John J. Pisano

Other Investigator: None

Cooperating Units: John Potts and Hugh Niall, Laboratory of Molecular Diseases, NHI

Man Years:

Total:	0.6
Professional:	0.6
Other:	0

Project Description:

The gas chromatographic method for the determination of the phenylthiohydantoin derivatives of amino acids has been further refined and applied to the determination of the structures of thyrocalcitonin and the parathyroid hormone. Refinements include the development of new column packings and the selection of packings for special problems. Application of the technique in the structural determination of thyrocalcitonin has been particularly gratifying as it was possible to verify the claims that this particular method is the most sensitive, rapid, quantitative and reliable.

Honors and Awards: None

Publications: None

Annual Report of the
ENDOCRINOLOGY BRANCH
National Heart Institute
July 1, 1967 through June 30, 1968

Studies in the Endocrinology Branch have included studies of adrenal function, of the physiology of bone, of renal function in health and disease, and of neuroendocrine relationships, as follows:

A. Studies of adrenal cortical function with special reference to biogenetic control mechanisms, inborn errors of steroid biogenesis such as the adrenogenital syndrome and the syndrome of juxtaglomerular hyperplasia without hypertension, and studies in the control of adrenal cortical secretion by peptides.

B. Studies of the physiology of bone with special reference to physicochemical factors controlling gain or loss of mineral from bone, effects of parathyroid hormone and of thyrocalcitonin of exogenous and endogenous origin in various disease states, measures of factors affecting absorption of calcium, direct measures of bone density as affected by hormones and disease.

C. Studies in renal function in health and disease, and of disorders in fluid and electrolyte balance. These have included extension and analysis of studies of idiopathic edema, studies of the role of the autonomic nervous system and of sodium-and-potassium-activated adenosine triphosphatase in sodium metabolism, studies of the renal circulation, and studies of the interaction of calcium and of sodium in the renal tubule.

D. Studies in neuroendocrine relationships included studies on the biochemical basis of taste and smell with especial reference to the role of copper and of Vitamin A and studies of the effect of steroid hormones on the central and peripheral nervous system.

A. Studies of adrenal cortical function. The role of potassium ions in the stimulation of adrenal cortical steroid production was explored by measurement of release of steroids in vitro from dog adrenal slices under the influence of previous sodium depletion, graded increases in ambient potassium ion concentration, steroid substrates and inhibitors of steroid oxidoreductases.

Earlier studies had indicated that sodium depletion affects steroid biogenesis at (at least) two sites, an "early" site - possibly that of side-chain cleavage of cholesterol - may depend upon angiotensin, which acts at this step; its stimulation was demonstrated by an increase of corticosterone production in salt depletion in vivo, and an increase in desoxycorticosterone secretion by these same adrenals from sodium-depleted animals in vitro when 11-hydroxylation was blocked by metyrapone.

The effect of potassium ions was studied in vivo and in vitro in similar preparations. In vivo, infusion of potassium ion increased the production of corticosterone and of aldosterone; cortisol production also often increased in this preparation. The results with "saturating" precursor substrate steroids in vitro indicate that the pathways from progesterone through desoxycorticosterone to aldosterone are potentiated by increases in potassium ion, but that cortisol production is not increased. Further, the conversion of corticosterone to aldosterone is not potentiated (and thus 18-hydroxylation is not stimulated), but the conversion of desoxycorticosterone to corticosterone by potassium is potentiated. The results thus support a site of action for potassium ion in the potentiation of 11 hydroxylation of desoxycorticosterone but probably not that of deoxycortisol.

With the same in vitro preparation two new inhibitors of 11- and 18-hydroxylation of steroids were explored. It was shown that the earlier results from in vitro studies - inhibition of 11 hydroxylase (desoxycorticosterone to corticosterone) and 18 hydroxylase (corticosterone to aldosterone) - could be confirmed in vivo at dosages not toxic to dogs. Thus, it is possible that the clinical application of such inhibitors to prevent peripheral action of steroid hypersecretion may become feasible.

Studies in the adrenogenital syndrome were extended. It was shown that whereas salt-losing congenital adrenal hyperplasia represents a biogenetic block in aldosterone secretion, probably dependent upon a defect in 21 hydroxylation of progesterone, no such block exists in the patients with non-salt-losing congenital adrenal hyperplasia, even though such patients clearly have a block in 21 hydroxylation of 17-hydroxyprogesterone, preventing the normal production of cortisol. Because of this block and of over-production of ACTH, these patients are hypersecretors of aldosterone. It was shown that control of aldosterone secretion was normal, responding normally to changes in the sodium intake, and that the hypersecretion could be reversed by inhibition of ACTH production through the use of carbohydrate-active steroids. Accordingly, it was postulated that 21 hydroxylase for progesterone and 17-hydroxyprogesterone are different enzymes or isoenzymes, and that the defects in the two types of patients are genetically distinct.

The syndrome of juxtaglomerular hyperplasia and normal blood pressure, with overproduction of renin and aldosterone was explored further. It was shown that the responsiveness to angiotensin, grossly subnormal in such patients, remained subnormal in the face of great expansion of intravascular volume and of sodium "space" by the infusion of albumin and salt. As these patients could also retain sodium quantitatively, it is clear that obligatory renal tubular sodium loss and hypovolemia are not essential features of the syndrome. Further studies are in progress to investigate further the control of renin secretion and of steroidogenesis by renin in patients with this syndrome.

Control of adrenal cortical steroid production by polypeptides was further explored. In the isolated adrenal cortex of the dog, a profound effect of lysine vasopressin on cortisol production was confirmed. This effect could

be obtained in hypophysectomized animals and thus must be considered independent of the reported action of vasopressin in potentiating steroidogenesis through the release of pituitary ACTH. Polypeptides having antigenic but no pressor activity are being studied for their ability to stimulate steroid production by the isolated adrenal in the hypophysectomized-nephrectomized dog. In this preparation, angiotensin II stimulates production of corticosterone, aldosterone and cortisol. As the clinical application of radioimmunoassay for angiotensin becomes prevalent, it is important that the relationship between antigenicity and steroidogenic potency of related peptides be clarified.

Studies on the control of renin secretion and the control of secretion of aldosterone and other sodium-retaining steroids in health and disease were extended. In balance studies in man, it was shown that body fluid volume (? intravascular volume) is more important than the serum sodium or osmolar concentration in control of renin secretion. The effect of adrenal disease on renin secretion was further explored. Earlier observations that there are syndromes of "primary" aldosterone over production, characterized by low renin production not responsive to salt deprivation or the upright posture, characterized by hyperplasia and not by tumor of the adrenal cortex was confirmed. As such patients developed all the signs of primary aldosteronism (hypokalemic alkalosis, hypertension) and produce low-normal quantities of cortisol and 17 ketosteroids, it is clear that a stimulus to aldosterone production which is not angiotensin or hyperkalemia or ACTH is operative. The possibility that in some such patients a metabolic block in steroidogenesis represents the initial event is being studied. For example, in two siblings with the syndrome of primary aldosteronism, very low production of aldosterone and of renin was demonstrated, indicating overproduction of sodium-retaining steroids in the aldosterone pathway, up to but not beyond the position of corticosterone.

B. Studies of the physiology of bone. The physico-chemical properties of bone were studied with the aid of bone itself (deprived of organic matter with ethylenediamine) and of a number of synthetic calcium phosphates resembling bone or the early products of calcification to a greater or lesser extent. By the use of radioactive calcium or of phosphorus in solution and by compartmental analysis of results, it was possible to examine separately factors affecting hydration shell and crystal surface and intracrystalline exchange. The model of a three-compartment system satisfactorily accounted for the results. It was shown that the solid phase of various calcium phosphates showed an affinity for accretion of calcium ions which is inversely proportional to the calcium-to-phosphorus ratio of the initial solid. Effects of various ions on bone marrow were further explored and studies with fluoride, sulfate and magnesium were added to those previously presented with sodium, potassium, lithium and cesium. The last four ions could be shown to substitute for calcium at the crystal surface on a molecule-for-molecule basis. Fluoride directly affected the activity of calcium ions in solution. Sulfate did not affect either solubility or mineral exchange, whereas magnesium could displace calcium ion from the bone surface. These studies are being extended to include ionic exchange with bones obtained clinically from patients

suffering from metabolic bone disease.

The relationship of activity product of calcium and phosphate in the urine to the formation of kidney stones was studied by direct determination of the activity product under a wide variety of conditions in which urinary calcium, phosphorus, sodium and pH were varied independently. With a direct method available for making such measurements. studies have been begun to identify or exclude the presence of unknown urinary substances which greatly effect solubility of bone minerals and of measures which are known or thought to be beneficial in the treatment of nephrolithiasis.

In a number of patients with kidney stones, the product was found to be abnormally high, as it was in patients with hypercalcemia of various origins. A number of measures currently employed for treatment of hypercalcemia were tested also for their effect on the solubility product. It was found that whereas phosphate markedly increased the product (despite its effect in lowering serum calcium), cellulose phosphate markedly decreased it, lowering serum and urinary calcium and magnesium. The effects of cellulose phosphate have been studied in a wide variety of patients. It appears on practical and theoretical grounds to be the most effective of available measures for lowering serum and urinary calcium.

Studies of the clinical disorder of osteoporosis were extended and further analyzed. Following "labelling" of bone with tetracycline and control measurement of microradiographic appearance of bone, patients with osteoporosis were given prolonged infusions of calcium which appeared to offer considerable clinical benefits. Calcium balance was reevaluated and the measurements of bone density and formation and destruction were repeated; in addition, the effects of calcium infusion were evaluated with Ca^{47} dynamics. The results suggest that calcium administered in this way may be of benefit. Studies are in progress designed to determine whether such benefit results from suppression of parathyroid hormone, or from stimulation of thyrocalcitonin.

Other patients with osteoporosis are receiving thyrocalcitonin of exogenous origin and the same variables are under study in these patients. As with infusion of calcium, the clinical response to thyrocalcitonin appears to be promising. It has been found that thyrocalcitonin can decrease urinary and serum calcium in adult patients and the results suggest that the effect derives from retention of calcium and phosphate in bone. The study of thyrocalcitonin effects in patients with hypercalcemia has been begun. In a patient with known carcinoma of the parathyroids, parathyroid hormone concentration in serum were shown to be constantly elevated and to arise in part from metastases in the lungs. The production of parathyroid hormone was not responsive to hypocalcemia induced with EDTA. Thyrocalcitonin in this patient reduced serum and urinary calcium, and induced a positive calcium balance. The apparent effect of thyrocalcitonin persisted for some weeks after treatment was stopped. With return of hypercalcemia some months later, a second course of thyrocalcitonin had the same effects.

Factors that affect the intestinal absorption of calcium were studied in extenso with the aid of a newly developed method for measuring absorption

by the trapping of absorbed ^{47}Ca in the bones of the hand and forearm. With this test, it was possible to divide patients with a history of renal stone formation into a group of hyperabsorbers and one with normal absorption. Evidence is accumulating that hyperabsorption is a familial disorder and a number of hyperabsorbers have been found among asymptomatic relatives of patients with persistent nephrolithiasis. It was shown that parathyroid extract promotes absorption of calcium from the gastrointestinal tract, except in patients with pseudohypoparathyroidism, and that Vitamin D has a similar effect. The effects of carbohydrate-active steroids of the adrenal cortex on calcium absorption are under study in patients with increased and decreased adrenocortical function and in normal subjects under different dosages of steroids.

Measurement of bone density in vivo by direct measurement of absorption of a monoenergetic photon beam was instituted and applied extensively to patients with and without metabolic bone disease. The method is reliable within $\pm$ 2% of the mean and is thus proving of value in the evaluation of therapeutic measures. For example, when calcium balance was improved with thyrocalcitonin in a patient with parathyroid carcinoma, the balance data could be immediately confirmed by direct densitometry.

Studies on the molecular forces involved in the interaction of hormones at interfaces were extended. It was found that by labelling the surface film with radioactive calcium small changes in the molecular organization of monolayer could be measured. With this preparation, it was found that insulin causes aggregation of the phosphate film and that the extent of this action suggests induction of miceller formation under the influence of insulin. With a monolayer of stearyl alcohol, sodium-retaining steroids were shown to lower the viscosity of the adherent liquid in the presence of potassium. With a layer of monooctadecyl phosphate, it was shown to increase viscosity, presumably also through action on the adherent liquid layer.

C. Studies in renal function in health and disease. In studies of the syndrome of idiopathic edema, a method for measuring 24-hour exchangeable sodium was developed and shown to give interchangeable results when estimated from total body counting, from balance of tracer and serum specific activity, or conventional balance studies. This method was applied to the patients with idiopathic edema and proved a much more sensitive index of total body sodium than was previously available. Some patients with idiopathic edema were found to suffer from obscure myocardiopathy such that end-diastolic pressures and pressure in response to exercise were below normal. The only manifestation of the disorder appeared as idiopathic edema and it could be remedied with digitalis. Other patients with a similar syndrome, having normal cardiac function, were shown to have abnormality of distribution of albumin with some decrease in circulating albumin and moderate to large increases above normal in the extravascular exchangeable albumin. In this group of patients, the aldosterone secretion rate was higher than in that of normals on a similar sodium intake and the response to aldactone consists of a much greater sodium loss than that of normals on the same regimen. In these patients the lymphatic vessels and their rate of removal of subcutaneously

administered albumin were normal. The abnormality of distribution of albumin involved primarily the "fast-exchanging" compartment thought to reflect visceral rather than peripheral sites. This group of patients thus appears to represent a new form of unexplained edema. A third group of patients were shown to have lymphatic hypoplasia. In this group the rate of removal of radioactive albumin from an extremity was markedly delayed. A moderate increase in aldosterone secretion and exaggerated response to aldactone suggests that the aldosterone production represents an attempt to compensate for loss of intravascular volume.

Studies of the role of the autonomic nervous system in the renal handling of sodium were continued in dogs with kidneys receiving all the renal blood from a donor, having thus only the renal nerve supply from the recipient. Hemorrhage in the recipient reduced its renal sodium excretion despite sodium loading of the donor. This supplies further proof that increased adrenergic activity can decrease renal sodium excretion.

Studies on the role of Na-K-activated adenosine triphosphatase on renal handling of sodium were begun in the rat. Whereas the evidence suggests that steroid-dependent sodium transport does not depend on this enzyme, the studies are designed to determine whether non-steroid-dependent sodium transport may be so controlled.

The renal blood flow was explored with the use of Xenon^{133} in dogs bearing isolated renal arteries; in some, constriction of the thoracic inferior vena cava was performed and urinary sodium excretion had decreased markedly. The results were subjected to extensive compartmental analysis and compared with simultaneous clearances. Whereas the results suggest that blood flow to the cortex and outer medulla is decreased by caval constriction, a "unique" solution could not be obtained by compartmental analysis and a new approach to mathematical models to analyze the circulation is underway.

The relationship of urinary calcium to urinary sodium was studied to clarify further the relationship between the renal transport of these two ions. The procedure of changing the tubular load of one ion by measures not known to affect the other has been employed. Early results suggest that whereas measures which increase sodium excretion, such as sodium loading and the use of aldactone, increase calcium excretion in parallel fashion, sodium retention can be induced without change in urinary calcium, and calcium loading can result in a dissociation of urinary calcium from sodium.

D. Studies in neuroendocrine relationships. In studies of neuroendocrine relationships it was found that patients who had been receiving penicillamine might present with marked decrease in sensitivity of taste. As the patients who showed this deficiency were also depleted of copper, the results suggest that copper is an essential in the taste mechanism. In some patients with the defect, administration of copper appeared to return taste sensitivity towards normal. Studies in rats were designed to test this hypothesis further. Rats, which would originally choose a solution of sodium chloride of varying concentrations, lost discrimination when treated with penicillamine and depleted

of copper. With continuing treatment with penicillamine and addition of copper, their taste discrimination returned. It was further found that monamine oxidase and proline hydroxylase activity are deficient in copper depletion and that these defects are related to the structural impairment of collagen under the influence of a copper-chelating agent. In rats given large doses of penicillamine, the defect extended to the central nervous system and the bones, producing a form of osteolathyrism. Defects of taste and of smell in the syndrome pseudohypoparathyroidism were further studied, as were those in a form of dysautonomia differing from the Reilly-Day syndrome in that apparently normal taste buds are present and no improvement is induced with methacholine.

Studies with Vitamin A suggest that this vitamin is required for mediation of the sense of smell. Thus, patients depleted of Vitamin A through malabsorption or other cause presented with hyposmia which could be greatly improved by administration of Vitamin A.

Studies of steroid hormones in the central nervous system included determination of the uptake into central nervous tissues of steroids administered intravenously. Whereas all steroids previously examined could readily enter central nervous system tissue, it was found that a large loading dose of cortisol given in advance of tracer prevented the uptake of cortisol by brain tissue. An interesting finding in the studies of central nervous system uptake of steroids appeared in the study of monkeys bearing transplantable brain tumors. It was found that the tumors take up radioactive cortisol in concentrations greatly exceeding those in the surrounding brain tissue.

The function of the steroids in living brain was further explored by measurement of cortical evoked potentials in patients lacking adrenal cortical function before and after treatment with cortisol. In contrast to the increase in sensitivity and in conduction rate found in adrenal cortical insufficiency, the manifestation of evoked potentials showed delay in the absence of carbohydrate-active steroids. Results thus suggest that synaptic transmission may be impaired by the same metabolic defect which may increase peripheral transmission, the combination leading to an information loss.

Serial No. NHI - 214
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Control of secretion of aldosterone and other adrenal steroids

Previous Serial Number: NHI-288

Principal Investigators: Burwell, L.R., M.D., and Bartter, F.C., M.D.

Other Investigators: Casper, A.G.T. and staff

Man Years:

Total:	.470
Professional:	.310
Others:	.160

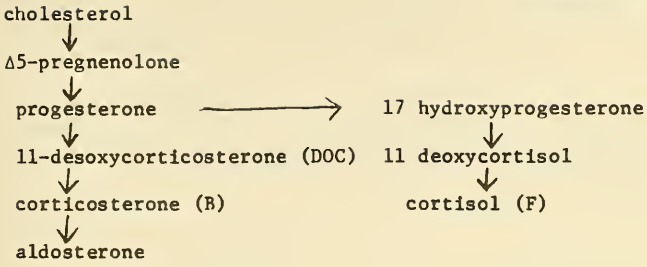
Project Description:

Objectives:

ACTH, angiotensin, depletion of body sodium, and increases of potassium in the incubation medium have been shown to stimulate the biosynthesis of aldosterone. ACTH and angiotensin increase aldosterone biosynthesis by stimulating the conversion of cholesterol to Δ-5 pregnenolone. Depletion of body sodium stimulates aldosterone biosynthesis by increasing the conversion of corticosterone to aldosterone and by stimulating steroidogenesis earlier in the pathway before the position of DOC. This earlier step may be mediated by the renin-angiotensin system. The present studies were to determine how increases in $[K^+]$ stimulate aldosterone biosynthesis.

Methods Employed:

Outer adrenal slices were prepared from hypophysectomized dogs which had been maintained on a 220 mEq Na^+ and 80 mEq K^+/day diet. These slices were incubated in Krebs-Ringer bicarbonate buffer of various potassium concentration, and the production of aldosterone, corticosterone, DOC and cortisol measured by the method of Kliman and Peterson. Non-radioactive and labelled precursor were used to define the locus of stimulation by elevated $[K^+]$.



Major Findings:

The production of aldosterone, B and F, from endogenous precursor is shown below.

	μg/g/2 hrs.		
$[K^+]$	Aldo	B	F
4.0	2.6 ± 0.3	1.0 ± 0.1	4.5 ± 0.8
8.0	3.3 ± 0.4	3.6 ± 0.3*	2.7 ± 0.3

* p <.001 vs. appropriate control

The stimulation of aldosterone biosynthesis by the high $[K^+]$ was found to be variable. Cortisol production was not affected. Corticosterone production, however, was consistently and significantly increased by high $[K^+]$.

To define further the locus at which increased $[K^+]$ acts, precursor studies were performed.

Table I - 1 μci H3 B as precursor

$[K^+]$ mEq/l	H^3 in Aldo DPM x 10^{-4}/100 μg/2 hr.	Mass of Aldo μg/g/hr.
3.8	11.1 ± 0.4	0.5
5.9	3.8 ± 0.5	1.6
8.3	3.0 ± 0.2	2.7

Table II - 1 μci H^3 B with 25 μg B

4.5	2.8 ± 0.6
8.5	2.0 ± 0.2

Increased $[K^+]$ in the medium did not stimulate the conversion of B to Aldo. This is in contrast to the stimulus of sodium depletion which persists for 3 hours of incubation and stimulates the conversion of B into aldosterone. The increased incorporation of H^3 B into aldosterone at a $[K^+]$ of 3.8 vs. 5.9 can be explained by dilution of the labelled B by non-radioactive B produced from endogenous precursors. Accordingly, the "pool" of B is larger at a $[K^+]$ of 5.9 and the added label represents a smaller fraction of that pool available to the enzyme.

579

Table III - 1 μci H^3 Progesterone as precursor for B

$[K^+]$	DPM x 10^{-5}/100 μg/2 hr.	
3.9	1.3 ± 0.14	p = 0.01
7.8	2.2 ± 0.24	

Table IV - Non-radioactive progesterone as a precursor for B

$[K^+]$ mEq/l	μg/g/2 hrs.	
4.0	15.7 ± 0.4	p <.001
8.0	25.4 ± 1.4	

Significant stimulation of the conversion of both tritiated and non-radioactive progesterone into B was observed at the high $[K^+]$ suggesting that stimulation occurs distal to progesterone in the pathway.

Table V - H^3 DOC as a precursor for B

$[K^+]$ mEq/l	DPM x 10^{-5}/100 mg/2 hr.	
4.0	3.2 ± 0.18	p = .02
8.0	4.3 ± 0.33	

Table VI - Non-radioactive DOC as a precursor for B

$[K^+]$ mEq/l	μg/g/2 hrs.	
4.0	25.8 ± 1.5	p <.01
8.0	33.2 ± 1.4	

These data indicate the conversion of DOC into B is stimulated by elevated $[K^+]$ in the incubation medium.

Additional studies are in progress to determine if the conversion of progesterone into DOC is affected by increase in $[K^+]$. Metyrapone, which inhibits the conversion of DOC into B, has been used to provide information about this earlier site. If more DOC accumulated in the high $[K^+]$ media, this would indicate stimulation in the pathway prior to DOC as well as at the step of DOC into B.

Publications:

1. Bartter, F.C.: Control of aldosterone secretion. In Gual, C. (Ed.): Proceedings of the Sixth Pan-American Congress of Endocrinology, Mexico City, Oct. 10-15, 1965. Amsterdam, Excerpta Medica Foundation, International Congress Series No. 112, 1966, pp. 97-103.

2. Bartter, F.C., Burwell, L.R., Davis, W.W., and Delea, C.S.: Control of the biogenesis of aldosterone. In Litonjua, A.D. (Ed.): Proceedings of the Third Asia and Oceania Congress of Endocrinology, Manila, Philippines, Jan. 2-6, 1967, pp. 184-194.

3. Ney, R.L., Dexter, R.N., Davis, W.W., and Garren, L.D.: A study of the mechanisms by which adrenocorticotropic hormone maintains adrenal steroidogenic responsiveness. J. Clin. Invest. 46: 1916-1924, Dec. 1967.

Serial No. NHI - 215
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of inhibition of adrenal steroid biosynthesis

Previous Serial Number: none

Principal Investigators: Burwell, L.R., M.D. and Bartter, F.C., M.D.

Other Investigators: Casper, A.G.T. and staff

Man Years:

Total:	.470
Professional:	.310
Others:	.160

Project Description:

Objectives:

GPA 1934 and GPA 2282 were synthesized by Geigy Chemical Corp. in an attempt to find a drug that would effectively inhibit aldosterone biosynthesis in vivo. Both compounds had been previously found to inhibit 11-hydroxylation (the conversion of 11-deoxycorticosterone to corticosterone and the conversion of 11-deoxycortisol to cortisol) and 18-hydroxylation (the final steps in the conversion of corticosterone to aldosterone) in bovine adrenal slices. In vivo and in vitro studies in the dog were undertaken to assess further the effectiveness of these agents.

Methods Employed:

In vivo studies were performed in intact dogs that had been depleted of body sodium by the administration of a mercurial diuretic two days prior to the experiment. The compounds were given orally, and the secretion rate of aldosterone, corticosterone, cortisol, and DOC measured from the lumbo-adrenal vein at hourly intervals after the administration of the drug.

In separate experiments, outer adrenal slices were prepared from dogs that had been hypophysectomized two days previously. These dogs were on a high sodium intake. The slices were incubated in Krebs-Ringer buffer with various concentrations of inhibitor. The steroids were measured by the method of Kliman and Peterson.

582

Major Findings:

The effect of 2 dose levels of GPA 2282 on adrenal steroid production in the intact dog is summarized below:

	mμg/min. Aldosterone	Corticosterone	DOC	Cortisol
Expt. 1 - 100 mg/Kg.				
Control	165	855	39	2300
1 hr.	25	798	33	1050
2 hr.	8	814	87	1190
3 hr.	13	872	70	1250
4 hr.	10	816	57	1370
Expt. 2 - 50 mg/Kg.				
Control	104	2006	19	2030
1 hr.	18	2464	35	2550
2 hr.	9	2596	150	1760
3 hr.	7	1854	77	1630
4 hr.	3	1138	38	1330

These data show that GPA 2282 is absorbed from the gastrointestinal tract, and that it inhibits 11-hydroxylation and 18-hydroxylation at both the 50 mg/Kg. and the 100 mg/Kg. dose level.

The steroid production rates by adrenal slices from a hypophysectomized dog maintained on a high sodium intake are shown below. The inhibitors were added in various concentrations. One unit of ACTH was added to each incubation flask to stimulate steroidogenesis.

	μg/g/2 hrs. Aldosterone	Corticosterone	11-Deoxycorticosterone	Cortisol
Control	1.3±.13	18.2±5.7	1.8±0.2	31.8
GPA 2282 0.4×10^{-4}M	0.5±.15	6.0±0.7	13.4±1.7	8.1
0.2×10^{-4}M	0.3±.1	6.0±0.8	7.5±0.8	10.3
0.04×10^{-4}M	0.5±.07	8.0±0.4	3.9±0.6	15.1
GPA 1934 1.0×10^{-4}M	0.4±.02	14.4±4.5		15.9
0.4×10^{-4}M	0.5±.11	14.7±4.7		14.5

GPA 1934, in the concentrations used, produced significant inhibition of aldosterone production. A slight inhibition of 11-hydroxylation was observed accounting for the decreased production of corticosterone. GPA 2282 produced approximately 60% inhibition in the synthesis of aldosterone, corticosterone, and cortisol. This compound has been shown to produce equal degrees of inhibition of 11-hydroxylation and 18-hydroxylation in bovine adrenal slices at concentrations of 0.4×10^{-4}M and 0.2×10^{-4}M. Additional studies are in progress to assess the effectiveness of GPA 1934 *in vivo* and the effect of both agents on the *in vitro* production of steroids by adrenal slices from sodium-depleted dogs.

None.

Serial No. NHI - 216
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the effects of infusions of fragments of Angiotensin II (A-II) upon aldosterone secretion of hypophysectomized-nephrectomized dogs.

Previous Serial Number: none.

Principal Investigators: Bravo, E.L., M.D., and Bartter, F.C., M.D.

Other Investigators: Casper, A.G.T. and staff

Man Years:

Total:	.440
Professional:	.390
Others:	.050

Project Description:

Objectives:

A direct correlation between circulating A-II and aldosterone secretory rate has not been demonstrated, partly perhaps because of imprecise assay methods for A-II. The advent of radioimmunoassay methods for A-II offers a more precise and specific procedure to determine circulating A-II in blood. In the process of its degradation by angiotensinases, the pressor and immunologic activities of A-II may be dissociated. Thus, it has been demonstrated that the heptapeptide 2-8 and the hexapeptide 3-8 fragments of the octapeptide A-II are immunologically active but deprived of pressor activity in physiologic amounts. It is not known whether the immunologically active A-II, stripped of its pressor activity, has any aldosterone-stimulating activity.

The present investigation has a two-fold purpose: 1) to determine whether the immunologically active, but physiologically inactive, fragments of A-II have any aldosterone-stimulating activity, and 2) to assess whether A-II, as determined by radioimmunoassay, can be correlated directly with aldosterone secretory rate.

Methods Employed:

Using the Hilton pouch preparation of the adrenal glands as described previously, the effects of infusions of the octapeptide A-II, heptapeptide 2-8, and hexapeptide 3-8, will be compared with regard to their pressor and aldosterone-stimulating properties.

Major Findings:

None at present.

Publications:

None.

Serial No. NHI - 217 (c)
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of steroidogenesis in the presence of high levels of circulating Angiotensin II

Previous Serial Number: none

Principal Investigators: Bravo, E.L., M.D., and Bartter, F.C., M.D.

Man Years:

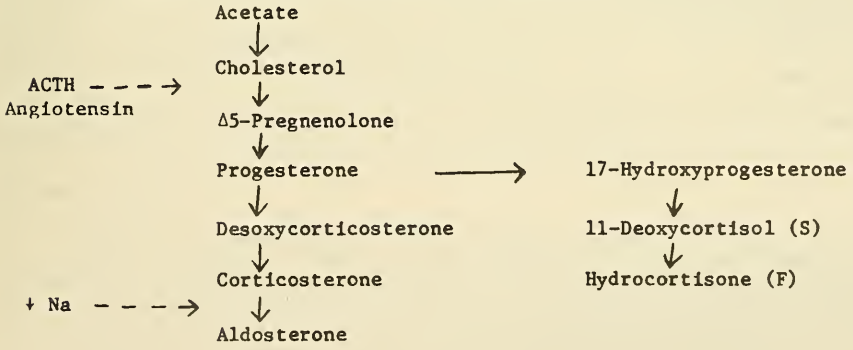
Total:	1.540
Professional:	.430
Others:	1.110

Project Description:

Objectives:

It has been established that aldosterone is synthesized by the adrenal cortex by a series of reactions that involve progesterone, deoxycorticosterone, and corticosterone as successive precursors.

Aldosterone biosynthesis and the sites of action of various stimuli
(ACTH, angiotensin, sodium deprivation)



587

Considerable evidence suggests that angiotensin acts at an early site in the biosynthetic pathways, most probably by activating the rate-limiting conversion of cholesterol to Δ5-pregnenolone, increasing the amount of precursor material available for the formation of corticosterone distal to this step, among them aldosterone. The hypothesis that aldosterone secretion rate is influenced by ACTH, by changes in renin and angiotensin, and by sodium metabolism have been well supported by experiments in the dog and sheep, as well as in man. The syndrome of juxtaglomerular hyperplasia with hyperaldosteronism, and normal blood pressure is of special interest in this context since it provides a situation in which the relationship between the renin-angiotensin system and aldosterone biosynthesis may be studied independent of the effect of ACTH and sodium metabolism. It has been established that in this syndrome the administration of dexamethasone and a normal salt intake do not affect the high levels of circulating Angiotensin II.

The experiment is designed to determine whether in man Angiotensin II acts early in the pathway of aldosterone biogenesis, so as to increase the biogenesis of aldosterone precursors or whether it acts late in this pathway merely to enhance the conversion of corticosterone to aldosterone.

Methods Employed:

The subjects of this study include patients with the syndrome of juxtaglomerular hyperplasia, hyperaldosteronism and normal blood pressure. They are maintained on a constant sodium intake under metabolic conditions. The ACTH-dependent production of corticosterone, deoxycorticosterone and cortisol is eliminated by the continuous administration of dexamethasone throughout this study. To employ deoxycorticosterone secretion rate as an index of intraadrenal formation of deoxycorticosterone, metopirone, a beta-hydroxylase inhibitor, is used. Secretion rates of corticosterone, cortisol, deoxycorticosterone and aldosterone are done at appropriate times during the study. Serum sodium and potassium and the urinary excretion of both electrolytes are followed through this study.

Major Findings:

None as yet.

Publications:

None.

Serial No. NHI - 218
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of infusions of lysine-vasopressin (LVP) upon cortisol secretion of hypophysectomized-nephrectomized dogs.

Previous Serial Number: none

Principal Investigators: Bravo, E.L., M.D. and Bartter, F.C., M.D.

Other Investigators: Casper, A.G.T. and staff

Man Years:

Total:	.440
Professional:	.390
Others:	.050

Project Description:

Objectives:

LVP has been used to assess the function of the anterior pituitary gland. More recently, it has been employed to distinguish between anterior pituitary and hypothalamic lesions. This is based on the assumption that LVP acts directly on the anterior pituitary gland. There are, however, somewhat conflicting reports regarding the mechanism whereby LVP causes a rise in cortisol production. LVP has been reported to have direct stimulating activity on the adrenals. Others claim that LVP acts directly on the hypothalamus to cause release of endogenous corticotrophin-releasing factor. Still others suggest that LVP acts directly on the pituitary gland.

The purpose of this study is to try to gain additional information on the mechanism whereby LVP increases cortisol production.

Methods Employed:

Normal adult, male, mongrel dogs, weighing between 20-30 Kg. were anesthetized by intravenous administration of 0.46-0.62 mg/Kg. body weight of sodium pentobarbital. The hypophysis was removed by the transbuccal approach. Both kidneys were removed and the adrenals were then prepared for perfusion by the technique of Hilton et al. Donor hypophysectomized-nephrectomized dogs were bled from the femoral artery the day before or the morning of the experiment. Blood was replaced volume per volume. Synthetic LVP was added to the arterial circuit leading to the adrenal glands at a rate of 1 ml/min. for 10 minutes. Concentrations used were 0.01 and 0.1 pressor units per ml. Blood

flow rate through the glands varied from 8-10 cc per minute during the experiment. Adrenal venous and arterial blood (30 cc. of each) were collected in graduated cylinders at the halfway point of the infusion period, rate of flow measured and the concentration of the steroids in plasma determined by a double isotope derivative assay.

Major Findings:

The first four infusion experiments have just been completed. Determination of the adrenal venous concentration of steroids is now underway. During the course of infusing LVP into the Hilton pouch preparation, there was a rise of about 20 mm Hg in the systemic blood pressure. Serum potassium and serum sodium remained constant during the experimental procedure.

The project will continue as outlined above. Modifications are anticipated in such areas as the time of sampling and concentration of LVP to be infused.

Serial No. NHI - 219
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies in calcium and phosphorus metabolism. I. Ionic interaction with bone mineral

Previous Serial Number: NHI-293

Principal Investigator: Pak, C.Y.C., M.D.

Other Investigator: Diller, E.

Cooperating Units: Skinner, C., Ph.D.,(Yale University,) Kempner, E., Ph.D., National Institute of Arthritis and Metabolic Diseases, and Weiss, M., B.S., National Institute of Arthritis and Metabolic Diseases.

Man Years:

Total:	.420
Professional:	.250
Others:	.170

Project Description:

Objectives:

The purpose of this project is to characterize by physiochemical means the various types of bone mineral and calcium phosphates. We shall show that the mineral phase of bone is a heterogenous system and varies widely with respect to chemical composition, calcium-to-phosphate molar ratio, solubility, and crystal size, as well as in their uptake of ions from solution. It is hoped that such a characterization will enhance the existing knowledge regarding the mechanism of pathologic calcifications and of ionic interaction with bone mineral in vivo.

Methods Employed:

The kinetics of uptake of tracer ^{45}Ca and ^{32}P by synthetic calcium phosphates and by mineral phase of bone was evaluated (method reported in publications 1 and 2). This permitted calculation of content of calcium and phosphate in the hydration shell of the crystal (adherent liquid layer).

Major Findings:

We have previously shown that the uptake of ^{45}Ca by hydroxyapatite or bone

591

powder could be reversed by wash-out into isotope-free solution containing stable calcium (publications 1 and 3). This constituted an experimental evidence for the isoionic calcium exchange process.

It was also shown that certain univalent cations (Na, K, Li, Cs) could substitute for calcium at the crystal surface on a one-to-one basis (publication 2). Larger cations (Tris and TMA) did not participate in such an interaction.

Subsequent studies have considered the following problems:

(A) Compartmental analysis of ionic exchange. The kinetics of the uptake of ^{45}Ca by hydroxyapatite was analyzed by fitting to 3-compartmental series model employing SAAM 24 program of Berman and associates. Compartment 1 is the ambient solution, compartment 2 the hydration shell, and compartment 3 represents the crystal surface. The physicochemical validity of this model was obtained. The intracrystalline exchange process was unaffected by stirring and ionic strength, but it rose with an increase in crystal surface area. The rate constants for the entry of radiocalcium into the hydration shell and crystal surface were influenced by ionic strength, surface area and rate of stirring. Further confirmation of this model was obtained from uptake of ^{131}Ba, ^{14}C, citrate, and ^{35}S sulfate. In contrast to ^{45}Ca and ^{32}P, these large ions did not participate in intracrystalline exchange.

(B) Physicochemical characterization of calcium phosphates. The exchange studies with ^{45}Ca and ^{32}P permitted calculation of the uptake of Ca(2+) and $PO_4(3-)$ from ambient solution by the solid phase. From the content of Ca (2+) and $PO_4(3-)$ in the hydration shell estimated from isotopic exchange studies, and from the initial and final concentration of Ca (2+) and $PO_4(3-)$ determined experimentally, the uptake by the solid phase alone, exclusive of the uptake by the hydration shell, could be calculated. The results show that synthetic calcium phosphates with low Ca/P ratio have a greater affinity for Ca(2+) than those with high Ca/P.

(C) Characterization of the mineral phase of bone. We have shown that the mineral phase of bone could be separated from the organic matrix by ethylenediamine extraction without significantly altering the mineral phase. The mineral phase from specimens of bone from patients with bone diseases, and from rats treated with various physiologically-active substances is being evaluated by chemical analysis, density, ionic exchange, solubility and x-ray diffraction.

(D) Effect of F(-), SO_4^{2-}, Mg(2+), and citrate on ionic interaction with bone mineral. Preliminary studies indicate that SO_4^{2-} has no effect on the solubility or the ^{45}Ca and ^{32}P exchange with the mineral phase of cow bone. F(-) markedly reduced the solubility, by principally affecting the concentration of Ca(2+) in solution. Mg(2+) increased the solubility, by displacing Ca(2+) from bone, while citrate increased the concentration of both Ca(2+) and PO_4^{3-} in solution.

(E) Metastatic calcification: calcium phosphate renal stones. The calcium phosphate customarily found in kidney stones is brushite ($CaHPO_4 \cdot 2H_2O$). The mechanism of formation and dissolution of brushite was examined in detail

in vitro. The activity product for Ca(2+) and HPO_4^{2-} was calculated at varying ionic strength.

The activity product of Ca(2+) and HPO_4^{2-} of normal urine generally falls below that of brushite, whereas the activity product of the urine of patients with calcium-containing stones exceeds the product of brushite. When the undersaturated urines are incubated with brushite, dissolution of brushite occurs. In contrast, the supersaturated urine (mostly from patients with stones) supports the growth of the synthetic stone (brushite).

This method should permit an examination of the factors involved in stone formation, as well as an evaluation of the efficacy of the various therapeutic regimen recommended for the control of calcium-containing renal stones.

Publications:

1. Pak, C.Y.C. and Bartter, F.C.: Ionic interaction with bone mineral. I. Evidence for an isoionic exchange with hydroxyapatite. Biochim. et Biophys. Acta 141: 401, 1967.

2. Pak, C.Y.C. and Bartter, F.C.: Ionic interaction with bone mineral. II. The control of calcium and phosphate exchange by univalent cation - calcium substitution at the hydroxyapatite crystal surface. Biochim et Biophys. Acta 141: 410, 1967.

3. Pak, C.Y.C. and Bartter, F.C.: Ionic interaction with bone mineral. III. Reversible calcium exchange with bone powder. Proc. Exp. Biol. and Med. 126: 126, 1967.

Serial No. NHI -220 (c)
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies in calcium and phosphorus metabolism. II. Treatment of patients with idiopathic osteoporosis with calcium infusions and with thyrocalcitonin (TCT)

Previous Serial Number: NHI-295(c)

Principal Investigators: Pak, C.Y.C., M.D., Evens, R., M.D. and Bartter, F.C., M.D.

Cooperating Units: Jowsey, J., Ph.D., Mayo Clinic, Rochester, Minn., and Hoye, R., M.D., National Cancer Institute.

Man Years:

Total:	2.205
Professional:	.765
Others:	1.440

Project Description:

Objectives:

Hypercalcemia induced by calcium infusion depresses parathyroid function and as a consequence decreases osteoclastic activity. Furthermore, hypercalcemia has been shown to stimulate the production of thyrocalcitonin (TCT) which inhibits resorption of bone.

In osteoporosis, an increase in bone resorption has been reported by histologic and isotopic methods. The long-term calcium infusion or the administration of TCT may therefore be beneficial in this disorder.

Methods Employed:

The patients were given 12-day course of calcium infusion (15 mg Ca/Kg/day) or TCT (2 MRC u. I.M./Kg/day). Before and after therapy, they underwent rib biopsies for microradiography of bone, $^{47}CaCl_2$ dynamics and calcium balance.

Major Findings:

(A) Calcium infusions. Four of five patients responded favorably to treatment. These four patients showed a reduction in bone resorption on microradiography, and an improvement in calcium balance ranging from 32 to 130 mg/day. Two patients showed an increase in the rate of calcium absorption from

5/4

the gut and in the rate of calcium accretion into bone. The fifth patient did not show any change on microradiography and in calcium balance. One patient was followed for 34 months after therapy; he continued to show an improvement in calcium balance.

Three other patients have undergone calcium infusions. Tentative results indicate a lowering of urinary calcium excretion and a slight fall in serum calcium with therapy.

(B) TCT. Only one patient has undergone the treatment with TCT. He responded by a fall in urinary calcium from control of 200 mg/day to 150 mg/day with treatment. His serum calcium also fell from 9.5-to 10 mg% to 8.8- to 9.3 mg%. These effects have persisted for three weeks after the suspension of therapy. The results of ^{47}Ca dynamics, complete calcium balance and microradiography of bone are not yet available.

Publications:

1. Pak, C.Y.C., Zisman, E., Lotz, M., and Bartter, F.C.: Gluconate carrier in ^{47}Ca kinetic studies. J. Clin. Endo. Metab. 27: 431, 1967.

Serial No. NHI -221 (c)
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies in calcium and phosphorus metabolism. III. Treatment of hypercalcemia with cellulose phosphate and thyrocalcitonin (TCT).

Previous Serial Number: None

Principal Investigators: Pak, C.Y.C., M.D., Wills, M., M.D., Delea, C.S. and Bartter, F.C., M.D.

Man Years:

Total:	1.715
Professional:	.765
Others:	.950

Project Description:

Objectives:

Treatment of hypercalcemia is often imperative because of the attendant nephrotoxicity and neuromuscular disturbance. A serious therapeutic dilemma may arise when the usual forms of therapy (e.g., sodium phytate, sodium phosphate, prednisone, or EDTA) are either ineffective or are contraindicated because of side-effects. The purpose of this study is to evaluate two new agents for the control of hypercalcemia, TCT and cellulose phosphate. Since TCT is believed to inhibit resorption of bone, it should be effective in hypercalcemia due to enhanced osteolysis. On the other hand, cellulose phosphate may be efficacious in conditions characterized by excessive gastrointestinal absorption of calcium.

Methods Employed:

A patient with hypercalcemia secondary to parathyroid carcinoma was given a 16-day course of TCT (2 MRC u/day) I.M. with gelatin carrier and another course without gelatin.

Two patients, one with sarcoidosis and another with parathyroid carcinoma, were given 10-to 20 gms. of cellulose phosphate per day orally.

Major Findings:

(A) TCT therapy. After 16-day course of TCT in gelatin carrier, there was a fall in serum calcium from 14.5 mg% to 11.9 mg%, with a concomitant

lowering of urinary calcium from 120- to 58 mg/day. After the suspension of therapy, there was a progressive fall in both serum and urinary calcium until they reached a low of 7.2 mg% and 36 mg/day respectively on the 20th day. There was also an improvement in calcium balance from -200 mg/day to +30 mg/day. The effect of TCT lasted 35 days.

When the patient was given TCT in saline carrier, there was a similar reduction in serum and urinary calcium during the period of therapy, but no sustained effect of the drug was observed after its suspension.

(B) Cellulose phosphate therapy. The administration of cellulose phosphate resulted in a fall in serum calcium to the normal range in 3-to 4 days. This was accompanied by a lowering of urinary calcium excretion and in serum magnesium. This was achieved by an inhibition of gastrointestinal absorption of calcium. Thus, the net calcium absorbed (V_a-EFC, or the amount of dietary calcium absorbed minus endogenous fecal calcium) was reduced by 67- to 123 mg/day with cellulose phosphate.

Unfortunately, this therapy did not influence the calcium loss; both patients continued to be in a negative calcium balance.

Publications:

None.

Serial No. NHI - 222 (c)
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies in calcium and phosphorous metabolism. IV. Intestinal calcium absorption in patients with renal calculi and other disorders of calcium metabolism.

Previous Serial Number: NHI-296(c)

Principal Investigators: Wills, M.R., M.D., Bravo, E., M.D., Pak, C.Y.C., M.D. and Bartter, F.C., M.D.

Other Investigators: Delea, C. and staff

Man Years:

Total:	.970
Professional:	.970
Others:	---

Project Description:

Objectives:

The purposes of this study are (a) to determine whether patients with renal calculi, with or without hypercalciuria, have an abnormal intestinal absorption of calcium and (b) to determine the intestinal absorption of calcium in patients with other disorders of calcium metabolism and (c) to assess the effect of parathyroid extract and Vitamin D on calcium absorption.

Methods Employed:

Gastrointestinal calcium absorption was determined by means of a simplified technique developed in our laboratory; this is based on hourly measurements of forearm radioactivity in a large sample scintillation counter after the oral administration of 2-4 microcuries of $^{47}CaCl_2$ with stable $CaCl_2$ as carrier. The values of forearm radioactivity are expressed as percent of the dose administered.

Major Findings:

(A) Patients with renal calculi. It has been previously shown in this laboratory that patients with nephrolithiasis have increased gastrointestinal calcium absorption when compared with normal subjects. The study has been extended to determine the effect of varying stable calcium loads over the range 20 - 1,000 mg. calcium as $CaCl_2$. Seventeen (17) patients with renal calculi

595

and four normal volunteers have been studied to date. Of the 17 patients, eight had hypercalciuria and nine were normocalciuric. Hypercalciuria was defined as a urinary calcium excretion of more than 200 mg. in 24 hours while on a diet containing 400 mg. calcium/day. To calculate the "absolute" amount of calcium absorbed from the oral load, each patient was also given an intravenous dose of ^{47}Ca and the forearm counted over the same period of time. The ratio of "% dose trapped after oral load" to "% dose trapped after intravenous load" was calculated and represents fractional calcium absorption. The product of the fractional calcium absorption by the stable oral calcium load gave the "absolute" amount of calcium absorbed at that load.

The values for the "absolute" amount of calcium absorbed at 4 hours after the oral load in the four normal volunteers are shown in Table 1 below:

	"Absolute" amount of calcium absorbed at stable calcium load.			
	20 mg.	200 mg.	500 mg.	1000 mg.
AT	15.0 mg.	76 mg.	213 mg.	247 mg.
S	17.9 mg.	115 mg.	193 mg.	267 mg.
GT	13.2 mg.	87 mg.	200 mg.	---
W	15.0 mg.	114 mg.	255 mg.	260 mg.

In each of the subjects studied there was increased calcium absorption with increased oral load.

The values for 9 patients with nephrolithiasis and normocalciuria are shown in Table 2 below:

	"Absolute" amount of calcium absorbed at stable calcium loads.			
	20 mg.	200 mg.	500 mg.	1000 mg.
EP	15.0 mg.	111 mg.	150 mg.	214 mg.
JW	9.9 mg.	76 mg.	159 mg.	249 mg.
LC	16.3 mg.	150 mg.	224 mg.	357 mg.
JD	12.4 mg.	105 mg.	146 mg.	283 mg.
HD	14.3 mg.	128 mg.	155 mg.	296 mg.
JM	13.8 mg.	120 mg.	149 mg.	239 mg.
BP	15.3 mg.	---	109 mg.	177 mg.
MR	13.9 mg.	69 mg.	123 mg.	---
CD	13.3 mg.	79 mg.	162 mg.	---

As in the normal subjects the "absolute" amount of calcium absorbed increased with the oral loading.

597

The values for eight patients with nephrolithiasis and hypercalciuria are shown in Table 3 below:

	"Absolute" amount of calcium absorbed at stable calcium loads.			
	20 mg.	200 mg.	500 mg.	1000 mg.
GP	20.0 mg.	153 mg.	317 mg.	---
AZ	17.9 mg.	143 mg.	213 mg.	284 mg.
JS	18. 2 mg.	150 mg.	263 mg.	399 mg.
EG	17.6 mg.	165 mg.	352 mg.	570 mg.
CS	18.8 mg.	200 mg.	---	508 mg.
RM	19.6 mg.	134 mg.	---	352 mg.
WB	19.1 mg.	94 mg.	184 mg.	---
SD	15.3 mg.	105 mg.	249 mg.	---

The results show, as in the other two groups, that calcium absorption increased with oral loading. In this group of patients, the "absolute" amount of calcium absorbed at any one load was significantly greater than in either the normal subjects or the patients with normocalciuria and nephrolithiasis.

(B) Patients with other disorders of calcium metabolism. Two patients with osteoporosis showed normal intestinal calcium absorption. One patient with osteomalacia of unknown etiology showed an abnormally low forearm radioactivity of 0.31% at one hour and 0.79% at four hours after the oral dose. A patient with hypoparathyroidism who was receiving insufficient doses of Vitamin D had low values of 0.37% and 0.73% at one and four hours respectively.

One patient with chronic renal failure showed low forearm radioactivity of 0.38% and 0.90% at one and four hours respectively. After 12 days of Vitamin D therapy 100,000 units per day orally, forearm radioactivity was unchanged with values of 0.34% and 0.84% at one and four hours. From intravenous ^{47}Ca data in this patient, the "absolute" absorption at four hours was 59 mg. and 55 mg. calcium before and after Vitamin D respectively, from an oral load of 180 mg. calcium.

Another patient with chronic renal failure showed low forearm radioactivity of 0.87% and 1.32% at one and four hours after oral ^{47}Ca, immediately prior to renal transplant. At three months after the transplant the values of forearm radioactivity were 0.88% and 1.82% at one and four hours. By five months post-transplant the values were 1.89% and 2.55% at one and four hours.

(C) The effects of Vitamin D and parathyroid extract. The effects of these substances which are known to affect calcium metabolism have been studied both in patients and in normal subjects.

Vitamin D.

The results of Vitamin D therapy are shown in Table 4 below:

Patient	Treatment	Serum Ca mg %	% Dose in Forearm after oral ^{47}Ca. 1 hr.	2 hr.	3 hr.	4 hr.
EO	Vit D 50,000 U q.d.	8.1	0.37	0.57	0.64	0.73
	Vit D 100,000 U q.d.	8.9	0.50	0.75	0.81	0.84
EC	No treatment	8.9	0.57	0.70	0.88	1.98
	AT 10 0.75 mg. q.d.	9.5	1.03	1.63	---	1.74
AM	No treatment	7.5	0.47	0.97	1.26	1.14
	Vit D 100,000 U q.d.	9.8	1.07	1.36	1.54	1.70
LE	No treatment	8.4	0.31	0.46	0.62	0.79
	Crystalline Dihydrotachysterol 1 mg. q.d.	9.5	0.40	0.68	0.82	1.01

Patients E.O., E.C. and A.M. had hypoparathyroidism and L.E. osteomalacia.

Parathyroid extract was given to 4 normal volunteers on a 580 mg. calcium metabolic diet in a dosage of 400 i.u. i.m. per day for 4 days. In all the subjects there was an increase in both serum concentration and urine calcium excretion. These changes were associated with an increase in gastrointestinal calcium absorption. The results of "absolute" calcium absorption from an oral load of 180 mg. calcium as calcium chloride are shown in Table 5 below:

	"Absolute" calcium absorption at 4 hours. Control	On Vitamin D
SS	103 mg.	123 mg.
CM	79 mg.	108 mg.
LB	102 mg.	116 mg.
MZ	103 mg.	121 mg.

The increases in urinary calcium excretion in these subjects are shown in Table 6 below:

	Urinary calcium excretion/24 hours	
	Mean 4 Control Days	Mean 4 days Vit. D
SS	165 mg.	284 mg.
CM	79 mg.	328 mg.
LB	115 mg.	272 mg.
MZ	121 mg.	205 mg.

The results show that the small but significant increase in gastrointestinal calcium absorption contributed to only a small part of the increased urinary calcium excretion.

(D) Patients with adrenal insufficiency. The pattern of calcium absorption in patients with adrenal insufficiency, on and off treatment, was determined. The test was done after four days on or four days off treatment with the patient receiving a diet with constant amounts of calcium (600 mg.), potassium, and sodium (109 mEq/day) and phosphorus. Some patients were placed on a constant metabolic diet to follow calcium, sodium, and phosphorus metabolism during the studies. Preliminary results on the first two patients are shown below.

J.D.

	Intravenous		Oral		Ratio: Oral/IV	
Hour	on Rx	off Rx	On Rx	Off Rx	On Rx	Off Rx
1	2.23	1.87	0.29	0.11	0.13	0.06
2	2.17	2.00	0.43	0.20	0.20	0.10
3	2.13	2.02	0.58	0.31	0.27	0.15
4	2.06	--	0.59	0.44	0.29	0.22

J.E.

	Intravenous		Oral		Ratio: Oral/IV	
Hour	on Rx	off Rx	On Rx	Off Rx	On Rx	Off Rx
1	2.76	2.14	0.28	0.46	0.10	0.21
2	2.29	2.19	0.39	0.72	0.17	0.34
3	2.29	2.27	0.44	0.85	0.19	0.37
4	2.31	2.23	0.54	0.92	0.23	0.41

Serial No. NHI - 223
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Interactions of hormones at the air-water interface

Previous Serial Number: NHI-294

Principal Investigators: Pak, C.Y.C., M.D., and Kafka, M.S., Ph.D.

Man Years:

Total:	.610
Professional:	.560
Others:	.050

Project Description:

Objectives:

The purpose of this project is to analyze the molecular forces involved in the interaction of hormones at the interface . Specifically, we have examined the interaction of insulin with phospholipid monolayer at the air-water interface, interaction of insulin monolayers with serum proteins, interaction of steroid hormones with lipid monolayers, and the interaction of Ca^{2+} with phospholipid monolayer.

Methods Employed:

The interaction of insulin with phospholipid and serum proteins was evaluated by observing the changes in surface area of the lipid film at constant surface pressure. The influence of steroid hormones on lipid monolayers was studied with surface viscosity. Finally the interaction of Ca^{2+} with phospholipid film was examined by the radioisotopic counting of the film. In the last technique, the interaction was studied by measuring changes in the radioisotopic count of the film. The lipid monolayer was spread over an aqueous buffer solution. A solution of ^{45}Calcium chloride and any substance(s) to be examined was added to the subsolution and the change in radioactivity over a given area measured by a Geiger-Mueller tube (D47 gas-flow detector, Nuclear Chicago) suspended at a fixed distance above the surface of the monolayer. Radioactivity measured before adding the ^{45}Calcium solution provided the background. The radioactivity over the area when ^{45}calcium solution was added to the subsolution minus the background radioactivity of that area provided a measure of the uptake of radioactivity by the film. The effect of insulin was shown by measuring the radioactivity of a monolayer spread over an aqueous subsolution to which 45Calcium and insulin were added and plotting it as a function of time. Appropriate controls were run.

603

Major Findings:

(A) When insulin (.1 mg/ml) is injected into the subsolution below the monomolecular layer of monooctadecyl phosphate at constant surface pressure, a marked decrease in film area is observed, suggesting that insulin causes aggregation of the phosphate film. This observation is compatible with the concept that insulin induces the micellar formation in the cell membrane.

(B) The insulin molecules from sheep, pork and beef are believed to differ only in the three amino acids in the Group A chain. Nevertheless, these insulins could be differentiated on the basis of their interaction with gamma globulin at the air-water interface (Publication 1).

(C) We have previously shown that sodium-retaining steroids lower the viscosity of the adherent liquid below the monolayer of stearyl alcohol in the presence of K^+, but not in the presence of Na^+. (Pak and Gershfeld, Nature 214: 888, 1967). In a further study, these steroid hormones were shown to markedly increase the viscoaity of monooctadecyl phosphate monolayer, after a delay of 5-10 minutes. Since a direct interaction of the steroid with the phospholipid was excluded, we must again conclude that the steroid hormone influence the hydrated region of the monolayer (Gershfeld and Pak, submitted for publication).

(D) Insulin in a concentration of 1 μg/ml subsolution inhibited the uptake of ^{45}calcium by the monolayer by 10%.

Publications:

1. Pak, C.Y.C. and Gershfeld, N.L.: Steroid hormones and monolayers. Nature 214: 888, 1967.

2. Arnold, J.D. and Pak, C.Y.C.: Interaction of soluble proteins with protein monolayers. J. Am. Oil Chem. Soc., 1967, in press.

Serial No. NHI - 224 (c)
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: In-vivo measurement of bone mineral content by a photon absorption method.

Previous Serial Number: None.

Principal Investigators: Evens, R.G., M.D., and Bartter, F.C., M.D.

Cooperating Units: Gurian, J., National Heart Institute

Man Years:

Total:	.440
Professional:	.390
Others:	.050

Project Description:

Objectives:

A reliable and accurate measure of mineral content of bone in the living subject is essential to the diagnosis and treatment of bone disease. Detection of diseases, such as osteoporosis and osteomalacia, characterized by radiolucency of the bones, and quantitation of bone mineral loss are difficult clinical problems. Evaluation of therapeutic measures is hampered by the lack of an accurate method of following the response in the patient.

Recent reports (Cameron, J.R. and Sorenson, J. Science 142:230-32, 1963, and Sorenson, J.A. and Cameron, J.R., J. Bone and Joint Surg. 49.-A: 481-497, 1967) have described an improved technique for measuring bone mineral content in vivo by mono-energetic photon beam transmission.

This laboratory has many projects underway in which a reproducible and accurate measure of bone mineral would be useful in the evaluation of therapeutic regimens for osteoporosis, osteomalacia, osteogenesis imperfecta and other metabolic bone diseases. Accordingly, this method was established and is currently in use for these tasks.

Methods Employed:

A photon source (100 millicuries of Iodine-125 adsorbed on Dowex ion exchange resin) is mechanically passed under the forearm, which is immersed in water. The collimated beam of photon is counted by a 1/2 inch by 2 mm. NaI (tl) crystal.

The crystal is connected to a multichannel analyzer and the pulses between 10 and fifty KeV are tabulated at 0.5-second intervals, which corresponds to the movement of the source and detector of 0.528 millimeters. The pulses are stored in the analyzer until the completion of a scan, which requires 128 channels or less. The dead time per channel is 14 microseconds, so that essentially all the pulses are tabulated.

The information is recorded on paper tape and analyzed by electronic computer (IBM 360) according to the formulations of Cameron and Sorenson. The mineral content and width of each bone in the path of the source and crystal are then calculated by the computer.

The mineral content is obtained from the computer in arbitrary units which represent the integral of the logarithims, $\Sigma \ln(IO/I)$. We have shown that these integral units may be accurately converted to grams of bone mineral per centimeter length of bone.

Major Findings:

Reproducibility

To test the reproducibility of the technique a dog femur, embedded in lucite, has been measured eleven times during a fourteen-week interval. The standard deviation was 2% of the mean.

Two separate determinations of bone density were made on eight normal subjects and two patients without active bone disease at intervals ranging from one to twelve weeks. The mean difference between the two values was 1% $\pm$ 1% (S.E.D.).

Normal Subjects

Determination of the bone mineral content of the radius has been performed on sixty-two normal subjects and patients without metabolic bone disease (Chart 1) in order to establish statistical limits of normal. This was accomplished with the aid of Miss Joan Gurian and tolerance limits according to the method of Wiessberg and Beatty (Technometrics 2: 483-500, 1960) were determined.

Patients with Metabolic Bone Disease

a) The bone mineral content of 22 patients with disease characterized by radiolucency of bones has been measured (Chart 2). A summary of these measurements follows:

Disease	Mineral content below normal range	Within normal range
Osteoporosis	6	1
Hyperparathyroidism	3	1
Osteogenesis imperfecta	2	--
Osteomalacia	4	--
Renal failure	2	--
Hypogonadism	2	--

b) Measurements have been performed on 6 patients with hypoparathyroidism (two idiopathic, three pseudo, and one pseudo-pseudo). Five of the values were within the normal range and the measurement on one patient was below normal.

c) Patients with recurrent renal stones. A summary of the measurements is as follows:

Urinary calcium	Bone mineral content below the range of normal	Bone mineral content within the range of normal
Normocalciuric	2	5
Hypercalciuric	--	4

d) This technique is currently being utilized to evaluate the treatment of osteoporosis, osteomalacia and osteogenesis imperfecta by obtaining serial measurements before, during and after various therapeutic measures.

Publications:

1. Evens, R.G., Ashburn, W., Pak, C.Y.C., and Bartter, F.C.: Clinical application of in-vivo measurement of bone mineral content by a photon absorption method. Conference on Progress in Methods of Bone Mineral Measurement. National Institutes of Health, Bethesda, Maryland. February, 1968.

Serial No. NIH - 225 (c)
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the interrelationship of the renal excretion of sodium and calcium.

Principal Investigators: Wills, M.R., M.D., Delea, C.S., and Bartter, F.C.,M.D.

Other Investigators: Casper, A.G.T. and staff

Man Years:

Total:	2.615
Professional:	.845
Others:	1.770

Project Description:

Objectives:

To evaluate the interrelationship of the renal handling of calcium and sodium and the mechanisms controlling this relationship. Earlier workers have claimed that these two ions are reabsorbed by a common tubular pathway and a direct relationship exists in the urinary excretion of these two ions.

Methods Employed:

Data have been collected from patients and normal subjects on metabolic regimen who have undergone therapeutic procedures that cause alterations in either urinary calcium or sodium excretion. The procedures during which data have been collected included calcium infusion, therapy with aldactone, 2 methyl-9α-fluorohydrocortisone and DOCA, and parathyroid extract administration.

Major Findings:

Calcium infusion. Results have been obtained in three patients with osteoporosis who were undergoing daily calcium infusions (15 mg/Kg. body weight i.v.) for 12 days. During a 12-day control period prior to the infusions the daily urinary calcium excretion expressed as mEq/24 hr. showed some correlation with urine sodium excretion. In all three patients on the first day of calcium infusion this relationship was maintained with an increase in urine sodium excretion correlating with the increased calcium excretion. After the first day of calcium infusion the increased urine calcium excretion was no longer correlated with increased sodium excretion and urinary excretion of the latter returned to values comparable to those obtained in the control period. During the 12-day period immediately following the calcium infusions when urine calcium excretion

had returned to normal values there was no correlation with sodium excretion.

Parathyroid extract. Four normal volunteers have been studied during the administration of parathyroid extract 400 i.v. i.m./day for four days. During a 4-day control period prior to parathyroid extract urinary sodium and calcium excretion showed some correlation. On the first day of parathyroid both urinary calcium and sodium excretion increased and the correlation was maintained. On the remaining three days the urinary sodium excretion fell while calcium excretion increased and the correlation was no longer maintained.

Aldactone. Three patients with idiopathic edema and two normal volunteers have been studied during the administration of Aldactone A, 200 mg/day for 8 days. During a 4-day control period prior to Aldactone there was a direct relationship between the urinary excretion of sodium and calcium. In all the subjects studied during the natriuresis associated with the administration of aldactone there was an increased calcium excretion and the relationship between sodium and calcium excretion was maintained.

Sodium-retaining steroids. Two subjects have been studied during the administration of 2 methyl-9α-fluorohydrocortisone 0.5 mg/day and one during DOCA 40 units i.m./day. In these subjects during the period of sodium retention associated with the therapy no change occurred in urinary calcium excretion, the calcium levels remain unchanged despite large changes in urinary sodium excretion. With the cessation of therapy there was a natriuresis and this was associated with marked increase in urinary calcium excretion, showing a direct correlation with sodium excretion.

The results in patients show that procedures which cause: 1. an increase in urinary sodium excretion are associated with an increase in urinary calcium excretion, 2. a decrease in urinary sodium excretion are not associated with a decrease in urinary calcium excretion, 3. an increase in urinary calcium excretion are associated with only a transient increase in urinary sodium excretion.

These observations are being extended by clearance studies in dogs during calcium infusion. The animals are being studied intact, after thyro-parathyroidectomy and after thyro-parathyroidectomy together with adrenalectomy. Nine clearance studies have been performed to date but no complete data are available.

Serial No. NHI - 226 (c)
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies in patients with "idiopathic" edema

Previous Serial Number: NHI-298

Principal Investigators: Gill, Jr., J.R., M.D., Evens, R.G., M.D., and Bartter, F.C., M.D.

Cooperating Units: Waldmann, T.A., National Cancer Institute

Man Years:

Total:	.770
Professional:	.720
Others:	.050

Project Description:

Objectives:

These studies were undertaken to determine the mechanism of sodium and water retention in women referred for evaluation of edema of unknown etiology.

Methods Employed:

The following studies were performed: right heart catheterization, lymphangiograms of the legs, the half-time rate of disappearance of subcutaneous ^{131}I - Albumin, aldosterone secretion rate (ASR) and treatment with aldactone, 200 mg. per day for six days. The results of these studies to date are summarized in the table below.

Major Findings:

	Cumulative Na loss with Aldactone mEq	Cumulative weight loss Kg.	ASR µg/day	T 1/2 subcutaneous ^{131}I-albumin hrs.
Normal	182	.79	55	15
Lymphatic hypoplasia	215	1.37	179	24
Cardiac abnormality	236	1.77	118	14
"Unexplained"	389	1.72	139	16

610

Hypoplasia of the lymphatic vessels of the legs was demonstrated by lymphangiogram; removal of subcutaneous albumin was delayed. The cardiac abnormalities consisted of pulmonary wedge pressure which was greater than normal at rest and remained elevated or increased further with supine leg exercise or was normal at rest and increased to an abnormal value with exercise. In some of these patients the right ventricular and diastolic pressure was also greater than normal.

In the patients labeled "unexplained", cardiac function was normal; lymphatic vessels appeared normal and subcutaneous albumin disappeared normally. In these patients, study of the intravascular albumin kinetics with ^{125}I-albumin in collaboration with Dr. Thomas Waldmann has shown a decrease in the amount of circulating albumin with an increase in the amount of extravascular albumin. The total albumin pool was normal or increased. The patients with cardiac abnormality and with lymphatic hypoplasia are being studied for the presence or absence of this abnormality in the distribution of albumin. Such an abnormality of distribution would result in a decrease in the extent by which plasma oncotic pressure exceeds extravascular oncotic pressure. This would tend to favor the movement of sodium and water from the circulation. The contracted plasma volume could increase the renal reabsorption of sodium through the increase in aldosterone secretion. The sodium diuresis with the aldosterone antagonist, aldactone, supports this hypothesis. The reason for the abnormal distribution of albumin is unknown.

Publications:

1. Bartter, F.C., Carr, A.A., Fleischmann, L.E., and Gill, Jr., J.R.: The role of the adrenergic nervous system in edema. In Gual, C. (Ed.): Proceedings of the Sixth Pan-American Congress of Endocrinology, Mexico City, Oct. 10-15, 1965. Amsterdam, Excerpta Medica Foundation, International Congress Series No. 112, 1966, pp. 225-258.

2. Gill, Jr., J.R., Bell, N.H. and Bartter, F.C.: Effect of parathyroid extract on magnesium excretion in man. J. Appl. Physiol. 22: 136-138, Jan. 1967.

3. Gill, Jr., J.R., Bell, N.H. and Bartter, F.C.: Impaired conservation of sodium and potassium in renal tubular acidosis and its correction by buffer anions. Clin. Sci. 33: 577-592, Dec. 1967.

4. Gill, Jr., J.R., Mason, D.T., and Bartter, F.C.: Effects of hydroxyamphetamine (Paredrine) on the function of the sympathetic nervous system in normotensive subjects. J. Pharmacol. Exp. Ther. 155: 288-295, Feb. 1967.

Serial No. NHI - 227
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the control of sodium excretion

Previous Serial Number: NHI-298

Principal Investigators: Gill, Jr., J.R., M.D. and Bartter, F.C., M.D.

Other Investigators: Casper, A.G.T.

Man Years:

Total:	1.715
Professional:	.765
Others:	.950

Project Description:

Objectives:

Previous studies from this laboratory showed that in dogs with constriction of the thoracic inferior vena cava and virtually complete renal reabsorption of sodium, ganglion blockade with pentolinium increased sodium excretion. This finding suggested that increased activity of the autonomic nervous system may be one of the means by which the organism decreases the renal excretion of sodium. The present studies were designed to obtain additional data on autonomic nervous system activity and renal function.

Methods Employed:

In these studies, the femoral artery and vein of a donor dog were connected by polyethylene tubing to a renal artery and vein of the recipient dog, whose kidney was thus perfused at femoral arterial pressure. The nerve supply to the perfused kidney was left intact. The donor dog was then infused with saline and urine collected by ureteral catheters from a kidney of the donor dog, the perfused and contralateral kidneys of the recipient dog. When urine flow in the donor dog and in the perfused kidney of the recipient dog reached maximal rates, the recipient dog was bled sufficiently to decrease mean arterial pressure 50 mm Hg, while continuing the infusion of saline in the donor dog.

Major Findings:

Regimen	Donor Dog Right kidney			Perfused kidney			Recipient Dog Contralateral kidney		
	$U_{Na}V$ mEq/min	$C_{\pm n}$	C_{PAH} ml/min	$U_{Na}V$ mEq/min	$C_{\pm n}$	C_{PAH} ml/min	$U_{Na}V$ mEq/min	$C_{\pm n}$	C_{PAH} ml/min
Control	33	29±3	66±5	13	16±4	38±8	12	30±4	80±9
Saline infusion donor dog	607	29±2	83±5	211	20±3	56±7	28	32±5	80±13
Hemorrhage recipient dog	556	28±2	75±4	124	19±3	45±6	6	12±3	41±9

Sodium excretion decreased in the perfused kidney when the recipient dog was bled, despite continuation of the saline infusion in the donor dog. The decrease in sodium excretion by the perfused kidney was apparently mediated by the renal nerves and was not associated with a change in the glomerular filtration rate. The findings suggest that increased adrenergic discharge to the kidney can lead to an increase in the tubular reabsorption of sodium. They support the notion that a decrease in the effective circulating blood volume may promote sodium conservation, at least in part, through an increase in adrenergic stimuli to the kidney.

Publications:

1. Gill, Jr., J.R., Carr, A.A., Fleischmann, L.E., Casper, A.G.T. and Bartter, F.C.: Effects of pentolinium on sodium excretion in dogs with constriction of the vena cava. Amer. J. Physiol. 212: 191-196, Jan. 1967.

Serial No. NHI - 228 (c)
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Twenty-four hour exchangeable sodium determinations in man.

Previous Serial Number: None.

Principal Investigators: Evens, R.G., M.D., Gill, Jr., J.R., M.D. and Bartter, F.C., M.D.

Man Years:

Total:	2.615
Professional:	.845
Others:	1.770

Project Description:

Objectives:

The determination of exchangeable sodium has been useful in the study of various diseases of sodium metabolism including a) edema-cardiac, lymphatic and idiopathic, b) psychiatric illness - mania and depression, c) juxta-glomerular hyperplasia, and d) congestive heart failure. We have evaluated the use of the whole body counter instead of urine collections in the determination of exchangeable sodium.

The technique of whole-body counting of clinically useful isotopes has the following advantages: 1) urine and stool collections are not necessary 2) unexplained losses are avoided, 3) measurement is easier, and 4) smaller doses of isotope may be used.

Determination of exchangeable sodium before and after manipulation of sodium balance (by diet, diuretic or sodium-retaining steroid) should be useful measurements of the effect of such agents in normal subjects and various disease states. We have compared the results of changes in sodium balance by standard balance techniques and exchangeable sodium measurements.

Methods Employed:

Twenty-four hour exchangeable sodium was calculated by dividing the amount retained after 24 hours, in counts per minute (cpm) or microcuries (μci) by the specific activity of serum. Amount of administered dose (25 μci ^{24}Na) retained was determined by two methods: 1) by subtracting total counts excreted in the urine from total counts administered, and 2) by whole-body counting. The patient was counted by using an 8 x 4 inch thallium-activated

sodium iodide crystal with fixed geometry in a low-background steel-shielded room.

Metabolic balance as performed in this laboratory includes chemical analysis of duplicate diets, confinement to air-conditioned areas and strict collection of urine and stool.

Major Findings:

The mean values for 24-hour exchangeable sodium in 55 studies by conventional urine and whole-body counting techniques were similar: 2374 mEq. and 2350 mEq., respectively ($P > .8$). The mean difference between the two techniques was 24 $\pm$ 13 mEq. (S.E.D.).

Twenty-four normals and patients had duplicate determinations of exchangeable sodium performed while on strict balance regimen and during various manipulations to alter sodium balance. The average change in sodium balance was minus 101 mEq. calculated from exchangeable sodium values and was minus 116 mEq. calculated from metabolic balance. The mean difference between the two techniques was 15 $\pm$ 22 mEq. (S.E.M.) and was not significant ($P > .8$).

These results demonstrate that the whole-body counting method, which may be performed periodically without the use of balance, gives reliable and reproducible data.

I. Comparison Urine vs. W.B.C. method.
A. Comparing actual values.

Group	No.	Mean	S.D.	S.E.M.	Coeff. Variation
Urine	55	2374.09	521.9	70.37	.2198
W.B.C.	55	2350.16	517.07	69.72	.2200

Mean Diff. $- 23.9273 \pm \frac{5.0}{9.9}$ S.E.D. $\frac{}{13.3}$

t ratio $- .241529$ (107.9 d.f.) $P > .8$

B. Comparing difference between 2 methods.

No.	Mean	S.D.	S.E.M.	Coeff. Variation
55	-16.5455	86.88	11.71	-5.25

$P > .2$ $t = \frac{\overline{x}}{S.E.M.} = 1.4$

II. Comparing Na_{ex} vs. metabolic balance

A.

Group	No.	Mean	S.D.	SEM	Coeff. Variation
Na_{ex}	26	-101.308	370.64	72.68	-3.65
Balance	26	-115.731	449.11	88.07	-3.88

Mean Diff. - 14.4231 $\pm$ S.D. 114.19 SEM 22.4

t ratio - .126297 (48.26 d.f.) P > .8

B. Comparing difference.

No.	Mean	S.D.	S.E.M.	Coeff. Variation
26	14.423	129.3	25.359	8.96

$$t = \frac{\bar{x}}{S.E.M.} = \frac{14.42}{25.359} = .568 \qquad P > .5$$

Serial No. NHI - 229
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Intrarenal hemodynamics as measured by Xenon^{133}

Previous Serial Number: NHI-299

Principal Investigators: Kafka, M.S., Ph.D., Newsome, H.H., M.D. and Bartter, F.C., M.D.

Other Investigators: Delea, C.S. and staff

Man Years:

Total:	1.22
Professional:	.80
Others:	.42

Project Description:

Objectives:

Under many physiological and pathological conditions, the changes in total renal blood flow and solute excretion might be explained by a modification in distribution of intrarenal blood flow. The project consists of a study of the effects of vena caval constriction.

Methods Employed:

Female mongrel dogs with urinary bladder trigones exteriorized and both renal arteries cannulated, were maintained on a diet constant in sodium and potassium and weighed daily. Group 1 (8 dogs) received no treatment, but 2 dogs of this group received antidiuretic hormone (ADH) on the day of the experiment; group 2 was adrenalectomized, treated with DOCA and cortisone and given ADH on the day of the experiment; group 3 was adrenalectomized, treated with adrenocortical steroids, received a partially constricting ligature around the thoracic inferior vena cava, and was given ADH on the day of the experiment.

The method of Thorburn et al was followed using Xenon^{133} in lieu of ^{85}Krypton. One to two weeks postoperatively dogs, deprived of food and water overnight, were anesthetized with sodium pentobarbital and their ureters catheterized. Before and during the period of Xenon^{133} injection, bilateral total urine collections were made and blood samples drawn for the measurement of standard clearances of inulin, p-aminohippurate, sodium, and potassium. The disappearance of Xenon^{133} injected into a renal artery was monitored over

that kidney by means of an iodide crystal scintillation probe.

Major Findings:

Recalculation and further analysis is now in progress to elucidate differences between groups of dogs studied with and without thoracic vena cava constriction.

Publications:

None.

Serial No. NHI - 229
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Intrarenal hemodynamics as measured by Xenon^{133}

Previous Serial Number: NHI-299

Principal Investigators: Kafka, M.S., Ph.D., Newsome, H.H., M.D. and Bartter, F.C., M.D.

Other Investigators: Delea, C.S. and staff

Man Years:

Total:	1.22
Professional:	.80
Others:	.42

Project Description:

Objectives:

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Methods Employed:

Female mongrel dogs with urinary bladder trigones exteriorized and both renal arteries cannulated, were maintained on a diet constant in sodium and potassium and weighed daily. Group 1 (8 dogs) received no treatment, but 2 dogs of this group received antidiuretic hormone (ADH) on the day of the experiment; group 2 was adrenalectomized, treated with DOCA and cortisone and given ADH on the day of the experiment; group 3 was adrenalectomized, treated with adrenocortical steroids, received a partially constricting ligature around the thoracic inferior vena cava, and was given ADH on the day of the experiment.

The method of Thorburn et al was followed using Xenon^{133} in lieu of ^{85}Krypton. One to two weeks postoperatively dogs, deprived of food and water overnight, were anesthetized with sodium pentobarbital and their ureters catheterized. Before and during the period of Xenon^{133} injection, bilateral total urine collections were made and blood samples drawn for the measurement of standard clearances of inulin, p-aminohippurate, sodium, and potassium. The disappearance of Xenon^{133} injected into a renal artery was monitored over

that kidney by means of an iodide crystal scintillation probe.

Major Findings:

Recalculation and further analysis is now in progress to elucidate differences between groups of dogs studied with and without thoracic vena cava constriction.

Publications:

None.

Serial No. NHI - 230
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of saline loading on rat renal cortical Na-K activated adenosine triphosphatase

Previous Serial Number: none.

Principal Investigator: Kallen, R., M.D.

Man Years:

Total:	1.230
Professional:	1.060
Others:	.170

Project Description:

Objectives:

The activity of rat renal cortical Na-K activated adenosine triphosphatase was examined as a possible metabolic correlate of the natriuresis induced by saline loading. Other workers have studied the effect of saline loading on proximal reabsorption by direct micropuncture technique, or assay of a concentrate derived from plasma of natriuretic rats by a nephron microperfusion technique. The present approach is designed to elucidate a possible metabolic concomitant of natriuresis; viz., an alteration in rat renal cortical Na-K activated adenosine triphosphatase activity. An alteration in activity of the enzyme might provide the basis for an assay of the natriuretic principle.

Major Findings:

The initial phase of the study was an attempt to characterize the enzyme prepared by the isolation technique of Chanock et al. The 41,000 xg and the 105,000 xg pellet of rat renal cortical tissue homogenate was assayed by the method of Chanock in both potassium-containing and potassium-free media, incubated for varying lengths of time (10, 20, 30 and 40 min). The 105,000 xg pellet was found to contain essentially no activity in contrast to the findings of others. The method is now being set up according to that of Katz and Epstein.

Serial No. NHI - 231 (c)
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Taste and Smell: Their relationship and mechanism in various disease states.

Previous Serial Number: NHI-303 (c)

Principal Investigator: Henkin, R.I., M.D.

Cooperating Units: Bosma, J.M., National Institute of Dental Research, Keiser, H., National Heart Institute, Scheinberg, I.H., Department of Medicine, Albert Einstein School of Medicine, Jaffe, R., Down State Medical College, New York, and Kare, M., North Carolina State College, Raleigh, N.C.

Man Years:

Total:	1.160
Professional:	.500
Others:	.660

Project Description:

Objectives:

These studies were undertaken to evaluate the relationship between the senses of taste and smell in various disease states. The studies during the past year have concerned themselves primarily with evaluation of the biochemical basis of gustation and olfaction.

Methods Employed:

These methods have been previously described, both in publication and in previous project reports. In addition, studies in animals were undertaken for the first time, during which preference thresholds were obtained in rats after treatment with various drugs.

Major Findings:

One of the major concepts which can now be added to the whole field of gustation is that copper may be a requirement in the taste process. In man, as discussed in previous project reports, and now in the rat, as presented in the report, copper appears to be the significant factor in normal gustation. In a series of studies carried out with the help of Dr. Keiser and Dr. Kare, rats were depleted of copper by administration of D-penicillamine and thresholds of

a preference type measured by the usual techniques. For example, given the choice between .15 molar sodium chloride and water, the control rats chose to drink sodium chloride approximately 70% of the time and to drink water 30% of the time. However, after treatment with D-penicillamine for approximately one month, rats preferred .15 molar sodium chloride 100% of the time. The same rats given a choice between .30 molar sodium chloride and water further established these differences. Normal rats reject .30 molar sodium chloride, taking it between 0 and 10% of the time and preferring water 90% to 100% of the time. Rats treated with D-Penicillamine, however, still preferred the .30 molar sodium chloride approximately 70% to 80% of the time. This indicates that their preference for salt is markedly affected when their serum and total body copper is depleted. Similar results have been observed for preference thresholds for sucrose. After treatment with a low dose of copper, .6 mg/Kg., there was some correction in the preference behavior of the D-penicillamine-treated rats. However, only after a high dose of copper was administered, 30 mg/Kg., did the taste response of the rats return to normal. At this time, it was not possible to distinguish between the rats treated with D-penicillamine and copper and the normal control rats. These results demonstrate that copper is necessary for the sense of taste, although its specific mechanism in this process is not yet clear. Studies regarding this mechanism are presently continuing.

In addition to these studies of taste, the effect of copper on various other systems has been evaluated. These studies, reported in detail by Dr. Keiser in his own project report, demonstrate that copper is critical in forming normal skin and that the enzymes monamine oxidase and proline hydroxylase are markedly inhibited in the copper-depleted animal. With the infusion of a small dose of copper, there is some induction of these enzymes and after the treatment of these animals with large doses of copper in addition to the D-penicillamine, enzymatic activity returns to normal levels. In addition, soluble collagen follows the same course. Other facets of the effects of copper depletion on rats have been observed in behavioral studies in which depleted rats manifest ataxia, neurological impairment of poor grooming, and movement of hind legs, in addition to the taste defects. Abnormally poor myelination of the spinal cord and brain, subperiosteal hemorrhages, and bowing of the long bones and spine such that these animals demonstrate osteolathyrism also occur. This is the first demonstration that copper deficiency produces osteolathyrism in rats.

In an effort to fully evaluate the effects of D-penicillamine on metabolism a new method innovated by myself and Silja Meret has been carried out, such that copper and zinc may be done on both plasma and urine simultaneously without precipitation of protein.

Studies in patients with various disease states have confirmed and extended our knowledge of the basic concepts of taste detection and perception. Four additional patients with the diagnosis of pseudohypoparathyroidism have been studied and a clear evaluation of the taste and olfaction defect in this syndrome has been made. It is now clear that some of these patients are indeed aware of their disability of both taste and olfaction and the genetics of this syndrome appear to be clearer. A new aspect of taste has been evaluated in a disease process which has been labeled Type II dysautonomia, or colloquially,

pseudodysautonomia. These patients have a similar taste defect to that of familial dysautonomia since they can neither detect nor recognize any concentration of solution of salt, bitter, sweet or sour. However, they differ from those patients with Type I familial dysautonomia (Riley-Day syndrome) in that examination of the tongue demonstrates what appear to be normal fungiform and circumvallate papillae. Indeed, linqual biopsies demonstrate normal taste buds in both the fungiform and circumvallate papillae. These have been evaluated by light and electron microscopy and cannot be distinguished from normal. Treatment of these patients with methacholine or acetylcholine does not in any way return their taste response to normal. This is in contrast to patients with the Riley-Day syndrome in whom treatment with these drugs does return taste responses to normal. This "end-organ defect" can be seen more clearly for in fact, not only do these patients demonstrate no triple response to histamine injection, but infusion with methacholine does not alter this abnormal response in any way. Again, this is in contrast to the abnormality seen in Type I familial dysautonomia, where the abnormal histamine response is corrected to normal with the parenteral infusion of methacholine. Thus, we have a new taste abnormality which in some way represents an end-organ resistance to acetylcholine which may or may not be missing from these patients.

Further studies in Vitamin A metabolism have corraborated and confirmed the studies noted in the previous project report which implicates Vitamin A as a major factor in the mediation of the sense of smell. Studies of numerous patients with defects of Vitamin A metabolism have revealed that each of these patients do indeed have deficient olfaction. Treatment of these patients with Vitamin A corrects their abnormalities somewhat and in some cases, corrects them to normal. A number of patients with what might be called "idiopathic hyposmia" have been studied during the past year and after a careful evaluation of their olfactory abnormality, they have been treated with Vitamin A. To this date, there has been some moderate improvement of olfaction, both in detection and recognition, in patients who are so treated, but their olfaction sensitivity has not yet returned to normal.

Publications:

1. Henkin, R.I.: Abnormalities of taste and olfaction in patients with chromatin-negative gonadal dysgenesis. J. Clin. Endocr. 27: 1436-1440, Oct. 1967.

2. Henkin, R.I.: Abnormalities of taste and olfaction in various disease states. In Kare, M.R. and Maller, O. (Eds.): The Chemical Senses and Nutrition. Baltimore, The Johns Hopkins Press, 1967, pp. 93-113.

3. Henkin, R.I.: The definition of primary and accessory areas of olfaction as the basis for a classification of decreased olfactory acuity. In Hayashi, T. (Ed.): Olfaction and Taste II. Proceedings of the Second International Symposium held in Tokyo, September, 1965. Oxford, Pergamon Press, 1967, pp. 235-252.

4. Henkin, R.I.: On the mechanism of the taste defect in familial dysautonomia. In Hayashi, T. (Ed.): Olfaction and Taste II. Proceedings of the Second International Symposium held in Tokyo, September, 1965. Oxford,

Pergamon Press, 1967, pp. 321-335.

5. Henkin, R.I.: Sensory mechanisms in familial dysautonomia. In Bosma, J.F. (Ed.): Symposium on Oral Sensation and Perception. Springfield, Ill. Charles C. Thomas, 1967, pp. 341-349.

6. Henkin, R.I., and Banks, V.: Tactile perception on the tongue, palate and hand of normal man. In Bosma, J.F. (Ed.): Symposium on Oral Sensation and Perception. Springfield, Ill. Charles C. Thomas, 1967, pp. 182-187.

7. Henkin, R.I. and Christiansen, R.L.: Taste localization on the tongue, palate and pharynx of normal man. J. Appl. Physiol. 22: 316-320, Feb. 1967.

8. Henkin, R.I., Keiser, H.R., Jaffe, I.A., Sternlieb, I., and Scheinberg, I.H.: Decreased taste sensitivity after D-penicillamine reversed by copper administration. Lancet 2: 1268-1271, Dec. 1967.

9. Henkin, R.I.: Abnormalities of the taste of sour and bitter and of olfaction in patients with pseudohypoparathyroidism. J. Clin. Endocr. In press, May 1968.

10. Henkin, R.I.: The role of taste and disease in nutrition. Bordon Review of Nutrition Research. Published by Bordon Co., New York, 1967 (invited paper).

11. Henkin, R.I., Christensen, R.L., and Bosma, J.F.: Facial hypoplasia, growth retardation, impairment of oral sensation and perception, and hyposmia: A new syndrome. Second Symposium on Oral Sensation and Perception, C. C. Thomas, Springfield, Ill., in press.

12. Henkin, R.I. Invited lectures to discuss taste and disease at the Pennsylvania Dietetic Association, spring meeting, and at the Virginia and West Virginia Dietetic Association, spring meeting. Also, a series of lectures will be given at the course in oral biology at the Massachusetts Institute of Technology, June 24-28, 1968.

Serial No. NHI - 232 (c)
1. Clinical Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effect of steroid hormones on peripheral and central nervous system activity and on the blood brain barrier in the cat and man.

Previous Serial Number: NHI-304 (c)

Principal Investigator: Henkin, R.I., M.D.

Cooperating Units: Walker, M.D., National Cancer Institute, Bethesda, Md., Daly, R.E., Johns Hopkins Hospital, Buchsbaum, M., National Institute of Mental Health, and Silverman, J., National Institute of Mental Health

Man Years:

Total:	1.490
Professional:	.830
Others:	.660

Project Description:

Objectives:

These studies are a continuation and extension of studies previously reported in the progress report ending June, 1967. The objectives of this year's studies were (1) to evaluate the dynamics by which cortisol and various other steroid hormones traverse the blood brain barrier of the cat, (2) to evaluate the manner by which cortisol is converted to cortisone in tissues of the central and peripheral nervous system of the cat, and (3) to evaluate the effects of endogenous and exogenous steroid hormones on various sensory and perceptual functions in man.

Methods Employed:

(1) The methods for evaluation of localization of cortisol, corticosterone and aldosterone have been described in previous project reports made by this investigator, and some of these have been published during this fiscal year. These publications will be listed at the end of this report.

(2) The methods for evaluation of transport of steroids across the blood brain barrier have been developed and extended during this past year. We have previously reported at the 50th Meeting of the Endocrine Society in Miami, June 1967, concerning the dynamics of passage of both cortisol and testosterone

across the blood brain barrier. Other hormones whose dynamics have been investigated include those of cholesterol, progesterone and estrogen.

New techniques have been developed to evaluate the binding and uptake of these hormones into the tissues of the cat. The cats were pre-loaded with non-radioactive cortisol and the dynamics of the uptake of tritium-labelled cortisol was evaluated in the same fashion, as has been studied previously.

The studies carried out in human subjects have been extended to include a number of sophisticated neurophysiological and psycho-physiological studies. With the help of Drs. Buchsbaum and Silverman visual and auditory cortical evoked potentials have been obtained in patients with adrenal cortical insuffiency, Cushing's syndrome and in normal volunteers receiving large dosages of exogenous cortisol during various aspects of their treatment programs. Cortical evoked potentials by the technique used in our laboratory represent a new method of studying the organization of the electroencephalogram since four stimulus intensities of light are used, which enables the investigator to study both amplitude and latency of the potentials in not just one, but in four quantitative steps. The normal pattern of response is correlated such that an increase in intensity of stimulation results in increase in amplitude and a shortening of latency of the evoked response. Thus, any deviation from this pattern is amplified by this quantitative stimulus response technique.

Major Findings:

In the cat after a loading dose of unlabeled cortisol, 20 mg/Kg., and waiting for a period of two hours for the drug to equilibrate, the tracer dose of tritiated cortisol was injected and biopsies of brain, plasma, CSF, muscle and various other tissues of the eviscerated cat were studied over an additional period of two hours. Contrary to our previous results, there was no uptake of the tritiated cortisol into the brain or into other tissues of the cat subsequent to the injection of the unlabeled material. In addition since there was no uptake of this material, no conversion of cortisol to cortisone was observed. The disappearance of the label from the plasma occurred at approximately the same rate as in the non-loading studies, but appearance in the cerebral spinal fluid occurred at a much more rapid rate than we had seen previously. This rate was so great that two hours after the injection of the single radioactive dose of cortisol, levels in the cerebral spinal fluid were approximately three times that noted in the brain. This observation has never been made previously. In an effort to quantitatively evaluate the blocking of uptake of radioactive cortisol into the brain and its appearance in the cerebral spinal fluid, a graded series of doses of unlabeled cortisol were introduced into the plasma of the cat. Thus, doses of 2, 0.2 and 0.02 mg/Kg. of "cold" cortisol were injected and after two hours an additional dose, this time of radioactive cortisol, was injected and its distribution measured over time. These studies demonstrated an almost linear response such that as the loading dose of cortisol decreased in concentration, the uptake of radioactive cortisol increased in all tissues. This was associated with the decrease in the concentration of cortisol found in the cerebral spinal fluid. At a dose of .2 mg/Kg., the amount of radioactive cortisol found in the brain was higher than that found in the cerebral spinal fluid. Similarly, as the uptake of the radioactive material was shown in the brain, there was a

subsequent conversion of cortisol to cortisone. Normally, one can expect, without the loading with cold cortisol, approximately 80% of the cortisol to be converted to cortisone in the brain. At the loading dose of .2 mg/Kg. with cold cortisol there was a conversion of only 30% of the radioactive cortisol to cortisone.

Additional studies utilizing similar experiments carried out in monkeys showed that after radioactive cortisol had been injected it could be followed by tracing the hormone in the various tissues of the monkey, including brain tumors. These monkeys maintained by Dr. Walker have a rather unusual abnormality; i.e., a brain tumor that has been kept viable in transplantation through a number of generations of monkeys. Injection of tritiated cortisol into the plasma of the monkey is followed by the appearance of this hormone in peripheral muscle, heart, lung, and other tissues in concentrations not unlike that seen in non-eviscerated cats. However, the appearance of the label in the tumor is approximately five to ten times that seen in the normal brain. Thus it appears that the radioactive hormone is concentrated in the brain tumor. In addition, conversion of cortisol to cortisone occurs in the brain tissue at a rate five to ten times more quickly than that observed in other tissues.

Sensory studies in human subjects have suggested that cortical evoked potentials measured by the techniques in the previous outline are significantly different in pattern in patients with adrenal cortical insufficiency off treatment than in these patients after they are treated with cortisol. Instead of having a consistent change in which increased amplitude and latency are found following increase in stimulus intensity, there was a decrease in latency and amplitude following an increase in stimulation. Thus, these patients are essentially "turning off" the incoming stimuli and inhibiting them, not only subjectively but as measured by cortical evoked potential activity.

Meaning of these experiments:

These experiments further demonstrate that adrenal cortical steroids play a significant role in brain function. (1) Cortisol crosses the blood brain barrier and is taken up in the brain. As previously shown, it is controlled in part by the presence of the adrenal glands. However, binding plays a significant role in the manner by which this hormone is taken up into the brain. (2) The enzyme for converting cortisol to cortisone is present in the brain and other tissues of the cat. Quantitatively, the presence of this hormone is perhaps in greater concentrations in the brain, determined by indirect methods, than in other tissues. Thus, approximately 80 to 100% of the cortisol can be converted to cortisone over time in the brain, while only 10 to 20% may be converted to cortisone in tissue such as muscle or fat. (3) The practical application of these techniques as in the study of brain tumors, and in their possible treatment, presents many unusual opportunities. It is clear that brain tumors, at least in the monkey, tend to selectively concentrate cortisol and this may prove useful in development of a therapeutic method. Its usefulness may be further accentuated since conversion of cortisol to cortisone also occurs at a more rapid rate in tumor tissue than in brain tissue. Thus, a relatively non-toxic radioactive hormone could be injected, accumulate in the brain and be converted to a more toxic metabolite. (4) Conclusions of studies done in human subjects indicates that there is a specific relationship between

detection and perception in the central nervous system, which is controlled, in large part, by the presence or absence of adrenal cortical hormones. This relationship seems to hold over numerous patients and in the number of situations other than adrenal cortical insufficiency. Into this relationship the model of how steroid hormones affect nervous system function has been made. The hypothesis that has been suggested and in some cases verified is that carbohydrate-active steroids normally act in the manner of a negative feedback in the nervous system such that incoming stimuli are inhibited and that stimuli which are transmitted are integrated optimally. When carbohydrate-active steroid is removed, sensory signals which are normally rejected are received by a sensory receptor, transmitted along the axon and to the central nervous system which tries to operate on these signals. Because of the changes in both peripheral nerve conduction and central synaptic transmission which occurs after removal of carbohydrate-active steroid, there is a marked alteration in the way in which these sensory signals are integrated. This results in an information loss. This model has been tested in a specific pharmacological situation in which LSD (lysergic acid diethylamide) has been given to a number of chronic users, studying a variety of sensory and perceptual tests. It is clear that these patients, during the effects of the drug, demonstrate marked increases in sensory sensitivity above normal, while at the same time, demonstrating marked disability in integration of sensory signals. This is particularly evident in the auditory system. When this drug is metabolized, approximately 24 hours after its administration, sensory detection and integration return toward more normal levels. However, in patients who are chronic users of this drug, these changes appear to persist such that sensory detection is more acute than normal, while sensory integration is poorer than normal. These results, as previously stated, support the hypothesis that there is a relationship between sensory detection and sensory integration in human beings.

Publications:

1. Henkin, R.I., Casper, A.G.T., Harlan, A. and Bartter, F.C.: The presence of cortisol and corticosterone in the peripheral and central nervous system tissues of the cat. Endocrinology, in press.

2. Henkin, R.I. and Bailey, R.E.: Auditory detection and perception in normal man and patients with adrenal cortical insufficiency: The role of adrenal corticosteroids. J. Clin. Invest., in press.

3. Ojemann, G.A. and Henkin, R.I.: Steroid dependent changes in human visual evoked potentials. Life Sci. 6: 327-334, Feb. 1967.

EXPERIMENTAL THERAPEUTICS BRANCH
NATIONAL HEART INSTITUTE

Basic and clinical studies conducted in the Branch during the past year will be considered under three headings: 1) Biochemistry and Pharmacology of Aromatic Amines, 2) Studies on Selected Proteins, and 3) Miscellaneous Observations.

BIOCHEMISTRY AND PHARMACOLOGY OF AROMATIC AMINES

A. Indoleamines. The tryptophan hydroxylase of rat brainstem has been purified about 4-fold using combinations of ammonium sulfate and column chromatographic fractionation. At this stage of purification the enzyme is soluble and clear in solution. The purified enzyme requires molecular oxygen and a reduced pteridine for activity. The enzyme activity is strongly stimulated by 2-mercaptoethanol, probably reflecting a protection of the co-factor. Since conflicting reports have appeared in the literature concerning the subcellular distribution of tryptophan hydroxylase in brainstem, this aspect of the problem has been re-investigated. When tissue is homogenized in 0.25 M sucrose, about 85% of the activity is found in the crude mitochondrial faction if the activity is assayed in the absence of co-factor and 2-mercaptoethanol. However, if the tissue is homogenized in hypotonic buffer and a fortified medium is used, most of the activity is in the supernatant fraction. While the soluble enzyme requires a reduced pteridine and 2-mercaptoethanol for activity, the particulate enzyme appears to be inhibited by these factors. For this and other reasons it appears that tryptophan hydroxylase may reside in a particle in the brain, the enzyme therein being easily solubilized. The soluble enzyme exhibits properties typical of an aromatic ring hydroxylase.

It was reported previously that p-chlorophenylalanine (PCP) and several catechol compounds are potent inhibitors of purified tryptophan hydroxylase. We have not found any additional potent inhibitors of the enzyme. Two metabolites of PCP, p-chlorophenylpyruvic acid and p-chlorophenylacetic acid were shown to be moderately potent inhibitors, being competitive with the substrate, and a number of halo-tryptophans (including the 6-chloro and 6-fluoro analogues) have been found to be weak inhibitors. Investigations have been extended on the effects of p-chlorophenylalanine in patients with the carcinoid syndrome. Studies in 11 patients permit the following tentative conclusions: PCP in daily oral doses of 2.0 to 4.0 gm results in a consistent decrease (50-80%) in the urinary excretion of 5-hydroxyindoleacetic acid; in 10 of the 11 patients the presumed decrease in 5-hydroxyindole production was accompanied by therapeutically-useful control of gastrointestinal symptoms, particularly diarrhea. In several patients there was nutritional benefit as evidenced by either a cessation of weight loss or an actual gain in weight which in one patient amounted to 25 pounds over a 3 month period. Our previous plan to study PCP in a variety of other clinical conditions has been cancelled because about 50% of carcinoid patients receiving the drug have developed a significant eosinophilia. While in our experience this has occurred in the absence of symptoms and is quickly reversible on stopping the drug, others have noted more severe accompaniments including urticuria, asthma and pulmonary infiltrates. Because of our initial observations in

5 patients that p-chlorophenylalanine therapy produced psychic effects, and in view of recent observations by Jouvet in France that serotonin depletion in the brain of cats, produced either neurosurgically or by administration of PCP, results in marked inhibition of sleep (insomnia), the effects of PCP on sleep "physiology" have been studied in 4 patients in cooperation with investigators of the NIMH. Although only mild central nervous system symptoms were observed with drug dosage up to 3-4 gm per day in these patients, striking changes in sleep pattern were recorded. These consisted primarily of a dose-related reduction in total sleep time and of rapid eye movement time (both absolute and as percentage) with a gradual return to control status over a period of 1 to 2 weeks following cessation of therapy. Additional studies are planned using agents such as 5-hydroxytryptophan which may be administered to replete brain serotonin, in an attempt to determine the specificity of the responses.

A better understanding of the rate of serotonin biosynthesis in vivo under various experimental conditions may be required for proper evaluation of the role of serotonin in the brain and other organs. Preliminary experiments in rats demonstrating easily measured incorporation of radioactivity into serotonin in brainstem, pineal gland and intestinal mucosa from 7-^{3}H-tryptophan injected intravenously suggests that it may soon be possible to assess serotonin synthesis rate and turnover in the intact animal. It is anticipated that functional correlations may thereby be established, which would not be revealed by existing techniques such as determination of tissue serotonin content.

An entirely different aspect of indoleamine physiology is implied by the observation of one of our investigators that pretreatment for 1 to 2 days with multiple injections of serotonin or tryptamine results in the development of significant tolerance (steroid-dependent) in rats to a variety of noxious stimuli, including tourniquet shock, endotoxin shock, anoxia, tumbling and exposure to cold. In the course of investigating the mechanism of this effect, a new apparatus was developed that permits continuous measurement of the oxygen consumption of the intact animal for prolonged periods and under a variety of ambient temperatures. Used in combination with cardiovascular monitoring techniques, detailed studies are in progress on the physiologic changes occurring following non-specific injury, alone and after indoleamine "immunization".

B. Catecholamines. In previous studies in collaboration chiefly with the LCB it was shown that the rate of synthesis of norepinephrine in sympathetically innervated tissues varies with nerve activity and that the regulation of norepinephrine synthesis involves end product inhibition at the tyrosine hydroxylase step. A variety of drug effects have been observed and reported upon previously. Recently, it was found that the administration of the adrenergic blocking agents, phentolamine (5 mg/kgm) and phenoxybenzamine (25 mg/kgm), results in a five-fold acceleration of epinephrine and norepinephrine synthesis from tyrosine-^{14}C in rats. The effect on rate of synthesis had the same time course as that of α-receptor blockade produced by these agents, being maximal when α-blockade was most pronounced and decreasing as the pharmacologic effect wore off. The increased synthesis rate was not demonstrable using ^{3}H-dopa as precursor, indicating that the effect occurred

at the tyrosine hydroxylase step. It is supposed that the decreased receptor responsiveness produced by the α-blocking agents results in enhanced adrenergic neural and hence biochemical activity possibly by a reflex mechanism.

A major orientation of catecholamine research in the Branch is toward a better understanding of the pathogenesis of various forms of hypertension in man and development of more effective therapeutic agents. Notably lacking in previous studies has been a suitable experimental model of human essential hypertension. In 1963 Okamoto and Aoki reported that they had produced a hypertensive strain of Wistar rats by selective in-breeding. The incidence of hypertension in these animals (referred to as "spontaneously hypertensive rats" or SHR) from the second generation onward has been 100%. A colony of SHR animals was established at the NIH with breeding stock brought to the Institute from Japan. The hypertension in these animals in some respects resembles human essential hypertension. It is not due to any of the recognized causes of secondary hypertension; the blood pressure increases progressively with age and in a considerable percentage of adult rats cardiac, renal and vascular complications occur. There is in these animals, as in patients with essential hypertension, an increased vascular responsiveness to norepinephrine. In our routine studies blood pressure is recorded in unanesthetized animals using a plethysmographic technique and is reported as systolic pressure. Blood pressure recorded, under anesthesia, directly from the femoral artery has correlated well with values determined by the indirect technique. We have confirmed that the systolic blood pressure of SHR animals is elevated at all ages studied (5-60 weeks), usually being, for example, 170 mm Hg at 15 weeks of age compared to 120 mm Hg in controls. A number of anti-adrenergic agents administered intraperitoneally produce a significant antihypertensive effect in the SHR animal. Drugs studied include methyldopa, chlorisondamine, α-methyl-tyrosine, pargyline and chlorothiazide. The antihypertensive effect observed with pargyline, a monoamine oxidase inhibitor, is noteworthy since, although drugs of this type produce obvious antihypertensive effects in human subjects, such an effect has not been demonstrated previously in an animal preparation.

In the course of studying a large number of O-methylated derivatives of phenolic phenylethylamines, Daly and associates in the Laboratory of Chemistry, NIAMD, discovered that 3,5-dihydroxy-4-methoxy-phenylethylamine was uniquely effective in depleting cardiac norepinephrine in the mouse. Further investigations have shown that the amine undergoes β-hydroxylation in heart tissue and that its chemical effects are consistent with a "false transmitter" mechanism. The latter constitutes the most popular concept of the mechanism of action of α-methyl-dopa, a compound whose antihypertensive effects were discovered in this Branch. In the case of methyl-dopa it is generally agreed that its biochemical and pharmacologic actions are owing in part to the fact that methyldopa enters sympathetic neurones and is converted by the intraneuronal, catecholamine synthetic machinery into the "false amines" α-methyldopamine and α-methyl-norepinephrine. It is supposed that the latter is released from the nerve endings during sympathetic nerve stimulation as a false transmitter, along with reduced amounts of the natural transmitter, norepinephrine. Probably the mechanism of action of methyldopa is more complex than this since α-methyl-norepinephrine is in itself a potent pressor substance whose action approaches that of norepinephrine. In contrast

to the α-methylated catecholamines, 3,5-dihydroxy-4-methoxy-phenylethylamine was found to be a very weak pressor substance being only about 100th as potent as α-methyl-dopamine. With the aforementioned principles in mind it was of obvious interest to study the biochemical and pharmacologic effects of the amino acid analogue, 3,5-dihydroxy-4-methoxy-phenylalanine (DHMPA). The compound was found to be notably lacking in toxicity with single doses as high as 1.6 gm/kgm producing no apparent gross effects in mice or rats. DHMPA was shown to be an effective depletor of the norepinephrine of heart, brain and spleen in the mouse and rat. Significant antihypertensive effects of the drug were observed in the SHR animal but not in normotensive controls. The potency of DHMPA, both as a catecholamine depletor and as an antihypertensive compound in the SHR animal, was about 5 times that of methyldopa. Extensive studies are underway on the mechanism of action of the drug in animals and preliminary clinical trials in hypertensive human subjects have been instituted.

5-Hydroxy-α-methyl-dl-tryptophan (α-methyl-5HTP) was studied in the Branch several years ago because of its properties as an inhibitor of aromatic amino acid decarboxylation. At that time single doses of the drug (200 mg/kgm) in the rat were shown to produce about a 50% depletion of cardiac catecholamines; this effect was interpreted as a reserpine-like action of the compound. Oral administration of up to 8 gm daily of α-methyl-5HTP to carcinoid patients failed to produce any effect, but this was shown to be the result of poor absorption of drug from the gastrointestinal tract. More recently Levitt and associates in LCB found α-methyl-5HTP to be a potent inhibitor of tyrosine hydroxylase. Furthermore, repeated dosing of animals with the compound resulted in profound tissue catecholamine depletion. The previous deterrent to further study of the compound, the poor absorption following oral administration, has been overcome by chemists at the Upjohn Co. who have synthesized the ethyl-ester of α-methyl-5HTP. The ester drug is effective by the oral route in animals both with respect to norepinephrine depletion and also in producing an antihypertensive effect in the SHR animal. Animal toxicity studies will be started shortly preparatory to administration of the compound to human subjects.

Another area of major investigation employing the SHR animal concerns the role of norepinephrine in the pathogenesis of experimental and clinical hypertension. While elevated blood pressure in patients with pheochromocytoma is clearly associated with increased production of catecholamines, attempts to discover a disturbance in catecholamine metabolism in other forms of hypertension have proved either negative or contradictory. The view that a disturbance in norepinephrine metabolism may account for various forms of hypertension in addition to that produced by pheochromocytoma has been strengthened by recent reports of deChamplain and associates in NIMH. They showed that animals made hypertensive with DOCA and salt have a disturbance in intraneuronal binding of norepinephrine which (they presume) leads to an increased "leakage" of the amine onto vascular receptors. We have felt that deChamplain's findings may not be applicable to human essential hypertension since the hypertension produced by DOCA-salt is acute in onset and associated with the rapid development of renal damage, fibrinoid change, necrotizing vasculitis and cardiac hypertrophy. Since hypertension in the SHR animal more closely resembles human essential hypertension it was of interest to investigate the state of catecholamine metabolism in this preparation. Initial studies were

detected by the platelet model. Anti-adrenergic drugs for which such an action has been proposed include debrisoquin, bethanidine, chlorobethanidine, guanethidine and bretylium. These drugs were administered to hypertensive patients in dosage sufficient to produce orthostatic lowering of blood pressure; laboratory investigation of bretylium and guanethidine is still in progress. Of the other compounds cited, debrisoquin was the only one found to inhibit platelet MAO in vivo. There were no associated changes in urinary amine excretion during treatment with this agent so that the thesis seems tenable that the anti-adrenergic effects of debrisoquin might be related to localized (intraneuronal) MAO inhibition. In these studies, the potentiation of the pressor response to tyramine was considerably less in intensity and duration than that generally associated with administration of potent MAO-inhibiting drugs.

STUDIES OF SELECTED PROTEINS

Both experimental and clinical studies of collagen metabolism have been continued using the biochemical indices described previously; these involve measurements of urinary and plasma hydroxyproline and a so-called "collagen profile". Two recent additions to the tissue assay profile are measurements of proline hydroxylase activity by methods developed in LCB and determination of collagen synthesis rate by a method recently developed in the Branch. The latter consists of incubating ^{14}C-proline with small tissue samples (e.g. dermal minces obtained by biopsy) and measurement of the rate of proline incorporation into collagen.

Under previous rationale, D-penicillamine in daily doses of 2-4 gm has been administered to a large number of patients with scleroderma for periods as long as one year. While definite increases in the percentage of acid extractable collagen were observed indicative of inhibition of collagen cross-linking in the dermis of these patients, the changes were of a much smaller magnitude than had been noted in patients with normal skin, during treatment with D-penicillamine. Thus, the biochemical effect of D-penicillamine on sclerodermatous skin has been less than might be expected to have therapeutic significance, and indeed no significant therapeutic effects have been noted by objective measures such as range of joint motion, pulmonary function, esophageal motility and body weight. Considering also the numerous toxic effects that occur during D-penicillamine therapy, there seems to be no reason for continued study of this drug in patients with scleroderma. An interesting by-product of the work was the first-recorded observation of a decrease in all modalities of taste concurrent with penicillamine therapy in some patients. This effect was most apparent in the decrease in taste acuity for sweet and salt. This abnormality was first detected by us in a patient who complained on the 22nd day of therapy that all food actually tasted salty. Questioning then revealed that she was adding large quantities of salt to her food and objective testing revealed a marked loss of taste sensation. An extensive study of the taste defect produced by D-penicillamine in man and animals has been completed in collaboration with Dr. Henkin of the CEB. Although chemical changes consistent with a lathyritic effect had been reported previously in animals treated with D-penicillamine, classic anatomic alterations of osteolathyrism had not been noted previously. However, in the course of the studies on taste in rats, it was found that animals receiving D-penicillamine in their

diet (10 mg/kg diet) consistently developed classic abnormalities of osteolathyrism, including kyphoscoliosis, exostoses and subperiosteal new bone formation. The collagen profile and the urinary excretion of hydroxyproline were also consistent with the development of osteolathyrism. The collagen abnormalities could be totally prevented or significantly ameliorated by the addition of copper (but not pyridoxine) to the diet.

The cross-linking mechanism in both collagen and elastin is presumed to be initiated by oxidation of specific epsilon amino groups of polypeptide lysine to form aldehydes which then undergo aldol condensation reactions to produce covalent bonds. We have presumed that the effect of D-penicillamine might be mediated by chelation of copper in a copper-containing amine oxidase. While this may still be true, the amine oxidase activity in skin as measured by oxidation of radioactive benzylamine, appears not to be markedly inhibited by the lathyritic agents, D-penicillamine and β-aminoproprionitrile. Furthermore, treatment of rats with a variety of monoamine oxidase inhibitors has not resulted in lathyritic effects; a possible exception is isoniazid. We have now tested a total of 21 different chemical compounds in a screening program in rats for effects on collagen cross-linking without discovery of any potentially useful lathyrogens.

In many studies on urinary hydroxyproline, it has been difficult to determine the degree to which changes observed represented alterations in collagen synthesis versus degradation rates. The proline incorporation technique mentioned above now shows promise of resolving some of these difficulties. Using dermal minces incubated in vitro it has been found that the rate of incorporation of proline into dermal collagen is much more rapid in material obtained from young, growing subjects (who presumably have a high synthesis rate) than it is in adult subjects. This is also apparent in studies on young and adult animals. A patient with active acromegaly was found to have a collagen synthesis rate comparable to that of a young, growing subject. No apparent differences have been noted in the collagen synthesis rates of skin from involved and uninvolved areas of patients with scleroderma.

Work has continued on the properties of the bacterial electron transfer proteins, ferredoxin and rubredoxin, with the specific goal of elucidating their catalytic centers. The role of the metal in these non-heme iron-containing proteins seems more generally relevant now since this type of protein is involved in a wide range of biological activities including dehydrogenation and steroid hydroxylation in mammals. We have isolated the non-heme iron protein of adrenal glands (termed "adrenodoxin") in milligram quantities from pig adrenals in order to study in detail its action on steroid hydroxylation.

It is now apparent that in both ferredoxin and rubredoxin the cysteines are not present as disulfides, but are indeed bonded to the iron that is present in the respective molecules. It is these iron-sulfur complexes that appear to form the catalytic redox sites in the protein. The artificial non-heme iron protein that is formed by reaction of bovine serum albumin with iron and inorganic sulfide in the presence of 2-mercaptoethanol is also being studied. This protein has a broad absorption spectrum similar to many of the native non-heme iron proteins. Our studies have provided convincing evidence

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that inorganic sulfide must be provided if the iron chromophore is to be formed. This is in contrast to a report by other workers that the inorganic sulfide arises via β-elimination of the polypeptide-bound cysteine. By use of optical rotatory despersion and circular dichroism techniques it was shown that the iron in the artificial non-heme iron protein is also bound to cysteine sulfhydryls.

MISCELLANEOUS

1. The results of further studies on the mechanism of inhibition of horse liver alcohol dehydrogenase by the thyroid hormones (thyroxine and triiodothyronine) indicate interference at the second step of coenzyme binding (i.e. binding of the nicotinamide portion of NADH) at or near the zinc site of the enzyme.

2. Preliminary studies on the metabolism of radioactive bradykinin proline-^{14}C in the second position) in human subjects revealed that about 10 per cent of an intravenous dose of radioactivity is excreted rapidly in the urine. About 80% of the radioactivity excreted appeared as a single peak on column chromatography; the nature of the peptide(s) is under investigation.

3. The discovery of patients with hypertension due to a deficiency of adrenal cortical 17-α-hydroxylase has stimulated our interest in corticosteroid synthetic mechanisms. Procedures have been set up using radioactive substances for in vitro study of the steps in adrenal steroid biosynthesis from cholesterol to corticosterone (via pregnenalone, progresterone and deoxycorticosterone). Initial application will be to the SHR animal and study of the effects of ACTH, 3', 5'-cyclic AMP and adrenodoxin. A search for 17-α-hydroxylase defects among cases of familial hypertension has begun (21 negatives to date) based on plasma assays for corticosterone.

4. α-Methyl-dopamine, a major metabolite of methyldopa, has been administered orally to hypertensive subjects, without consequent cardiovascular effects. Further evaluation for possible antihypertensive effects does not seem warranted in view of known pressor potency of the compound on intravenous administration.

Serial No. -NHI-233
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Biosynthesis and Metabolism of Physiologically Active Amines.

Previous Serial Number: NHI-168

Principal Investigator: Walter Lovenberg, Ph.D.

Other Investigators: Albert Sjoerdsma, M.D., Ph.D., Donald Robinson, M.D. and G.H. Besselaar, M.D.

Man Years

Total: 1.15
Professional: 1.15
Other: 0

Project Description:

Objectives: Serotonin is a physiologically active substance that has been proposed as a neurotransmittor. The objective of this project is to study its synthesis in detail and determine what factors may affect its rate of synthesis in vivo.

Since tryptophan hydroxylase is the rate limiting enzyme in serotonin biosynthesis the efforts are directed toward a thorough characterization of this enzyme.

Methods: Two methods have been used to measure tryptophan hydroxylase. The first of these developed in this laboratory and was used to measure hydroxylase activity in homogenates or soluble enzyme preparations. It consist of incubating the enzyme with radioactive tryptophan in the presence of trapping amounts of 5-hydroxytryptophan (5 HTP); after completion of the incubation the reaction is treated with purified aromatic L amino acid decarboxylase. The serotonin formed is isolated and its specific radioactivity determined. From this amount of tryptophan hydroxylated can be calculated. The second procedure was recently reported by Hakanson and is based on the conversion of ^{14}C tryptophan to ^{14}C-5HTP in the presence of the decarboxylase inhibitor, NSD-1055. The radioactive 5HTP is then separated from the substrate by thin layer chromatography. This assay was found to be particularly useful in measuring hydroxylation in whole pineal gland of rat and mucosal cells obtained from the intestine. Serotonin synthesis rates in vivo were studied

using radioactive tryptophan given I.V. to rats. Serotonin was isolated from various tissues by butanol extraction followed by chromatography on thin layer.

Major Findings: Tryptophan hydroxylase has been purified from rat brain stem about 4-fold with a 30% recovery of total activity. The purification steps were: ammonium sulfate fractionation followed by adsorption and elution from calcium phosphate gel, and refractionation with ammonium sulfate. The enzyme appeared to be soluble and completely clear in solution. This purified enzyme solution required molecular oxygen and a reduced pteridine for activity. Although 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$) was used routinely as the cofactor, tetrahydrobiopterin was much more effective as a cofactor (activity 2 to 3 times greater and K_m 1/50). The enzyme activity was strongly stimulated by 2-mercaptoethanol probably reflecting a protection of the cofactor. Although ferrous iron stimulated the tryptophan hydroxylase isolated from pineal it had no appreciable effect on the brain enzyme. Both enzymes were strongly inhibited by α,α-dipyridyl, an iron chelator. Of interest was the finding that not only would ferrous iron reverse the inhibition of α,α-dipyridyl, but a two-fold increase in activity over the initial activity was observed indicating that the enzyme activity may be inhibited by trace metals that are present in the crude enzyme. The pH optimum for the purified brain enzyme appeared to be about 7.6. Since tryptophan hydroxylating enzymes in mammalian tissues have many properties in common with liver phenylalanine hydroxylase it was of interest to compare phenylalanine: tryptophan hydroxylase activity of enzyme preparations from several tissues. The ratios are as follows: rat liver = 10:1, mouse mast cell = 2:1, beef pineal = 1:1, and rat brain stem = < 0.1:1. While it is not known whether both these hydroxylase activities are catalyzed by a single enzyme in any of these tissues it is indeed possible that tryptophan and (or) phenylalanine hydroxylase exists in several molecular forms in mammals.

A number of compounds were examined as inhibitors of the purified tryptophan hydroxylase. Catechol compounds were rather potent inhibitors if the enzyme was assayed in the absence of exogenous Fe. Norepinephrine, dopamine, dopa, α-methyldopa and catechol, all exhibited 65-75% inhibition when present in incubation mixture at 10^{-4} M. Kinetic studies indicated that the catechol compounds were typical non-competitive inhibitors with regard to either the substrate or cofactors. The inhibition could be partially reversed by the addition of Fe^{++}. Phenylalanine, p-chlorophenylalanine, p-chlorophenylpyruvic acid and p-chlorophenylactic acid were moderately good inhibitors being competitive with the substrate. A number of halo-tryptophans were examined for inhibition. 6-Chloro and 6-fluoro-tryptophan were weak inhibitors (50% inhibition at 10^{-3} M).

Since conflicting reports have appeared in the literature concerning the subcellular distribution of tryptophan hydroxylase in brain stem, this aspect of the problem has been reinvestigated. When the tissue is homogenized in 0.25 M sucrose, about 85% of the activity is found in the crude mitochondrial fraction if the activity is assayed in the absence of cofactor and 2-mercapto-

ethanol. If however the tissue is homogenized in hypotonic buffer and the fractions assayed in a fortified medium the majority of the activity is in the mitochondrial supernatant fraction. Examination of the enzyme activity in the soluble and particulate source showed that the soluble enzyme required DMPH4 and 2-mercaptoethanol for activity whereas the particulate enzyme appears to be inhibited by these factors. From this study and the work of Grahame-Smith et al (Biochem. J. 105, 351, 1961) it would appear that tryptophan hydroxylase may reside in a nerve-ending like particle in the brain, but this enzyme is easily solubilized and in the soluble form shows the typical properties of an aromatic ring hydroxylase.

Using the Hakanson method of assay it has been found that the tryptophan hydroxylase activity in pineal incubated in vitro (0.5 to 1.0 mμmole 5HTP/pineal/2 hours) is two to four times higher than comparable amounts of homogenate assayed under optimal conditions for the cell free enzyme. Para-chlorophenylalanine (PCP) which is competitive with substrate in the soluble enzyme also exhibited similar inhibition with the whole pineal. It was found, however, that pineals from rats treated with PCP (300 mg/kg) showed little or no inhibition. This finding is in contrast to the apparent irreversible inhibition that occurs in rat brain stem.

Low tryptophan hydroxylase activity has also been detected in mucosal cells from the small intestine of the rat (0.005 mμmole 5HTP per mg tissue per 2 hours). Comparing the synthetic rate with the known serotonin content in this tissue permits the calculation of serotonin half-life in the intestine (15 hours). This is in good agreement with previously proposed values.

Preliminary studies on the in vivo synthesis indicate that tryptophan is poorly incorporated into serotonin when given intravenously. Injection of 20 μc of ^{14}C tryptophan into the tail vein of a monoamine oxidase-inhibited rat resulted in the incorporation of less than 1000 dpm/gm tissue into the serotonin of either brain or intestine. No other tissues contained significant label in serotonin.

Significance: A complete understanding of the mechanisms involved in serotonin synthesis appears to be essential for evaluating the physiological role of serotonin. The presence of both serotonin and norepinephrine in many tissues suggest a possible interaction between these two amines. This is particularly important in view of the strong inhibitory properties of the catecholamines on tryptophan hydroxylase. Other compounds which were first studied in the above in vitro system such as PCP have proved to be useful in regulating overproduction of hydroxyindoles in man.

Proposed Course of Project: The future studies will be directed toward developing techniques for measuring in vivo serotonin synthesis in various tissues and comparing this with the results in vitro. A study of the effect of various compounds on the rate of serotonin synthesis in whole animals will be done. Work on the purification and characterization of tryptophan hydroxylase in various tissues will continue.

Honors and Awards: None

Publications:

1. Lovenberg, W.: Aromatic L amino acid decarboxylase. S. Colowich and N.O. Kaplan (Ed.) Academic Press, New York. In press.

2. Lovenberg, W., Jequier, E. and Sjoerdsma, A.: Tryptophan hydroxylation in mammalian systems. Advances in Pharmacology. In press.

3. Robinson, D.S., Lovenberg, W. and Sjoerdsma, A.: Subcellular distribution and properties of rat brain stem tryptophan hydroxylase. Arch. Biochem. Biophys. 123: 419-420, Feb. 1968.

4. Jequier, E., Lovenberg, W. and Sjoerdsma, A.: Tryptophan hydroxylase inhibition: the mechanism by which p-chlorophenylalanine depletes rat brain serotonin. Mol. Pharmacol. 3: 274-278, April 1967.

Serial No. -NHI-234 (c)
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies related to the Carcinoid Syndrome

Previous Serial Number: NHI - 166 (c)

Principal Investigator: Karl Engelman, M.D.

Other Investigators: Albert Sjoerdsma, M.D., Twitty Carpenter, M.D. (NIMH) and Richard Wyatt, M.D. (NIMH).

Cooperating Units: Adult Psychiatry Branch, NIMH

Man Years

Total: 1.05
Professional: .55
Other: .5

Patient Days: 800

Project Description:

Objectives: Investigation of the carcinoid syndrome has continued with primary efforts involved in extending our earlier preliminary findings on the effects of inhibiting serotonin synthesis with the new drug - parachlorophenylalanine (PCP). Recent clinical studies have substantiated our earlier impression of the beneficial effects derived from this drug on the diarrhea of the patients with the carcinoid syndrome, and studies are now being focused on the role of PCP in producing altered personality and sleep patterns in patients receiving the drug. The studies of personality changes during PCP administration are an outgrowth of our initial findings that patients receiving the drug appeared to become more anxious and to have certain other neurological and personality changes. The sleep studies, however, were undertaken following reports that PCP when administered to cats produced an altered sleep pattern and insomnia. It was thought to be important to document whether these changes occur in man and to attempt to derive an explanation of the changes on the basis of biochemical changes produced by the drug.

Methods: In addition to our standard biochemical methods for the qualitative and quantitative analysis of indole compounds in blood, urine and tissues, we have begun studies requiring new techniques for our unit. In cooperation with co-investigators from the National Institutes of Mental Health we have been studying the sleep pattern of patients before, during and following administration of PCP. This is done by recording the electrical impulses from the

electroencephalogram, oculomotor muscles and from the muscles of the submandibular areas during the entire sleep cycle. Analysis is then made of the duration and depth of sleep, the periodicity and duration of the rapid eye movement (REM) phase, and other changes which allow the sleep physiologist to evaluate neurophysiological changes produced by PCP. In order to better evaluate any psychiatric changes produced by PCP, more intensive personality evaluation has been undertaken with the aid of a psychiatrist and an experienced nurse. Daily mood evaluations made by these observers in addition to frequent self-evaluations performed by the patient (mood-evaluation questionnaire) form the basis of the appraisal of psychiatric changes in these subjects.

Major Findings: Evaluation of PCP administration to a total of eleven patients with the carcinoid syndrome permits certain preliminary conclusions. The biochemical effects of PCP on serotonin production, as reflected by the excretion of 5-hydroxy-indole-acetic acid (5HIAA) in the urine, were found to result in consistent reductions of from 50-80% on daily doses of 2.0 - 4.0 gms. This reduction of hydroxyindole production resulted (in 10 of 11 patients) in marked reduction in both upper and lower gastrointestinal symptoms. Diarrhea was completely stopped in six of the patients and daily stool weight was also reduced. Two patients actually required laxatives or enemas because of the development of constipation while receiving PCP. In several patients there was either a cessation of weight loss or actual weight gain while receiving the drug and one patient gained 25 pounds during three months of therapy.

Though experience in the first five patients receiving PCP indicated a high incidence of psychiatric changes, the patients treated subsequently have only developed mild symptoms when the dose was increased to 3.0 to 4.0 gms per day. Changes in sleep pattern have been observed in each of the four patients in whom sleep recordings have been made. There is a striking dose-related reduction of total sleep time and of the REM time during therapy and return to control values after cessation of therapy is slow, taking up to two weeks.

Proposed Course of Project: During the past year it has become evident that significant eosinophilia develops in about 50 per cent of patients receiving PCP. Though we have experienced no greater toxicity, others have noted urticaria, cough, pulmonary infiltrates and even asthma in patients receiving PCP. All those changes are reversed within several days of stopping the drug. Because of these findings further studies with PCP are being confined to patients with malignant carcinoid disease, and we will be unable to study the effect of this drug in either normal subjects or in patients with other diseases such as migraine headache and sprue as originally planned.

Despite this limitation we plan studies in an attempt to replete brain serotonin with either tryptophan or preferably 5-OH-tryptophan in an attempt to determine whether the altered sleep pattern and personality changes are due to serotonin depletion in the central nervous system.

Honors and Awards: None

642

Publications:

1. Engelman, K., Lovenberg, W. and Sjoerdsma, A.: Inhibition of serotonin synthesis by p-chlorophenylalanine in carcinoid syndrome. New. Eng. J. Med. 277: 1103-1110, Nov. 1967.

Serial No.-NHI-235
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Physiological Chemistry of Serotonin and Tryptamine

Previous Serial Number: NHI - 173

Principal Investigator: W.D. Denckla, M.D.

Other Investigators: None

Man Years

Total: .7
Professional: .5
Other: .2

Project Description:

Objectives: To determine why these indolalkylamines are markedly more toxic for adrenalectomized rats compared to intact rats and how the rat which is intact or which is adrenalectomized and receives cortisone builds tolerance to this amines in 24-48 hours.

Methods: Cardiac output was determined with a BL 610 electromagnetic flow-meter placed directly on the ascending aorta after thoracotomy. Ventilation was maintained with a rodent respirator (Harvard). Drugs were infused via the femoral veins and blood pressure was recorded via the femoral artery. Probes were calibrated in vitro with saline infusions through the aorta post-mortem.

Major Findings: It was found that both tryptamine and serotonin caused a decrease in cardiac output and that this decrease was not as pronounced when rats had been made tolerant to the indoleamines by pretreatment with tryptamine. The cardiac output of the untreated rats decreased to 40-50% of control values at the same dose of serotonin that caused a decrease to only 75-80% of control values in the pretreated animals. Under the experimental conditions this difference in response was similar to the difference in toxicity between the pretreated and control animals (2 fold). Adrenalectomized rats appeared to be appropriately even more sensitive than the intact control rats to the decrease in cardiac output caused by the amines. The infusion rate varied from 5-30 μgm 5HT/kg/min. The effective tryptamine pretreatment schedule was 50 mg/kg four times a day for one to two days i.p.

644

Significance: Since there are no reports on the effects of serotonin or tryptamine on cardiac output in rodents, this work illuminates in a unique way a major pharmacological effect of these amines which has not been previously investigated. The altered toxicity in adrenalectomized rats, it is hoped will help to elucidate some of the mechanism underlying the vascular changes in such animals.

Proposed Course of Project: To identify the specific intravascular site and mechanism for the increased toxicity, the changes in cardiac output and to try and determine using oxygen consumption measurements during the infusion of 5 HT or tryptamine if the nutrient microcirculation is affected as badly as the macrocirculation.

Honors and Awards: None

Publications: None

Serial No. -NHI-236
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigations into the Endocrinology and Physiology of Non-Specific Vascular Resistance to Injury

Previous Serial Number: None

Principal Investigator: W. D. Denckla, M.D.

Other Investigators: None

Man Years

Total: .7
Professional: .5
Other: .2

Project Description:

Objectives: In the literature over the last 40 years various reports have been made as to the development of tolerance to injurious agents such as tourniquet shock, endotoxin shock, anoxia, tumbling, cold and unspecifically injurious chemicals such as formaldehyde. It has been further reported that a certain amount of cross protection occurs when animals, (rats have been used for the most part), are made tolerant to one agent. It is a matter of some controversy whether such tolerance can develop in adrenalectomized rats. Even those who report tolerance developing in adrenalectomized rats found considerably less resistance to the injurious agent than found in intact rats. Most authors agree that glucocorticoids are either absolutely more beneficial or relatively more beneficial in restoring the mechanism which permits the development of tolerance when compared to mineralocorticoids. In the case of cold adaption no one at the present has ever even questioned whether a thyroidectomized rat could adapt to cold. This mechanism appeared to offer a tool to investigate the basis of the generally more sensitive vascular system of the adrenalectomized rat and to determine the cause of this increased sensitivity to virtually all forms of injury.

Methods: Two entirely different techniques were used to test for the development of tolerance, exposure to cold and administration of tryptamine. Tolerance was developed in rats acutely by the exposure of the rat to these "agents" over 1-4 days. Thyroidectomized rats were also tested along with adrenalectomized and adrenalectomized-gonadectomized rats. Appropriate varying dosages of replacement hormones were given. The major end-points measured were the response in the cold as measured by alterations in oxygen consumption and alterations in the LD 50. To measure the oxygen consumption at various

496

environmental temperatures a ten channel digital recording metabolic unit was developed utilizing a closed circuit system with a spirometer and a photochopper transducer.

Major Findings: It was found that perfect cross tolerance could be demonstrated between those rats which were maximally resistant to tryptamine and those which had been made resistant to cold by exposure to cold. It was further found that the tolerance could develop to approximately 50% of maximum to either stressor in the absence of the adrenal and that corticosterone and cortisone restored fully maximal tolerance. Mineralocorticoids (DOCA) appeared to be ineffective when given at reasonable pharmacological doses i.e. 2 mg/kg. Removal of the gonads restored some tolerance in the adrenalectomized female. The hormone responsible for some of the deleterious vascular effects in the female appears at present to be estrogenic and not progestational in nature. While removal of the thyroid diminished the basal metabolic rate and made such rats very sensitive to cold, pretreatment with cold exposure permitted such animals to have the same per cent rise in oxygen consumption in the cold as the euthyroid controls i.e. 100%. Since even with this increase in metabolism the cold-tolerant athyreotic rats were unable to offset heat losses in the cold, these rats remained more sensitive to the cold compared to their controls. This is the first albeit preliminary demonstration of cold adaptation occurring in athyreotic animals.

Significance: This work appears to clarify some of the major mechanism required both for non-specific vascular tolerance and the development of tolerance to cold. Tolerance to cold appears to require: 1) thyroid for heat production, 2) the sympathetic nervous system to make appropriate cardiovascular alteration consequent to increased metabolism in cold, 3) adrenal to prevent vascular collapse due to cold injury and 4) non-specific vascular resistance to prevent ischemic and decreased nutrient flow in the cold-injured tissues. Non-specific vascular resistance appears to develop independently of the adrenal or the thyroid, but in the absence of these glands and depending on the "stressor", full physiological resistance is not obtained.

Proposed Course of Project: There is a strong suggestion that the peripheral vascular system is adapting to the constrictor effects of the endogenously released catecholamines possibly by altering the levels of monoamine oxidase in the tissues. All current evidence as to the time course of the development and disappearance of this protective mechanism indicates a possible enzymatic alteration in the animal. The physiology of this mechanism and its related endocrinology opens a large vista for exploration since it appears to involve some of the major endocrine systems of the body as well as some of the major organs. Future physiological experiments will be directed toward defining more precisely where in the body the adaption takes place and to try and understand the biochemical basis of this adaption. To this end the following preparations have been developed: a system for measuring ventricular function curves in the rat with which one can vary load and flow independently as well as determine the left ventricular end diastolic pressure, a system for the measurement of VO_2 in the anesthetized rat with a

time constant of 15 seconds and an accuracy of 1%. These preparations and related techniques will permit a quantitative examination of this mechanism and the interrelationships of the endocrine, cardiovascular and autonomic nervous systems.

Honors and Awards: None

Publications: None

Serial No. -NHI-237
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Isolation and Characterization of Clostridial Electron Transfer Proteins and Other Non Heme Iron Proteins

Previous Serial Number: NHI - 167

Principal Investigator: Walter Lovenberg, Ph.D.

Cooperating Units: Central Research Dept. of DuPont, Wilmington, Delaware
University of Washington, Seattle, Washington.

Man Years

Total: 1.9
Professional: .4
Other: 1.5

Project Description:

Objectives: The goal of the project is to elucidate the catalytic centers of the small electron transfer proteins, rubredoxin and ferredoxin, and to study their relationship to biological activity. It is anticipated that findings in these studies will facilitate understanding the role of the metal in many other non-heme iron-containing proteins. Many of these proteins presumably have iron chromophores which are similar to that of ferredoxin. This type of protein is involved in a wide range of biological activity including dehydrogenation and steroid hydroxylation in mammals, photosynthesis in plants and nitrogen fixation and hydrogenase linked reactions in bacteria. While little is known of the role of rubredoxin it has been shown to serve as the reductant for the mixed function oxidase that catalyzes the hydroxylation of alkanes in Pseudomonas oleovans.

A variety of chemical and physical techniques have been employed to probe the nature of the iron chromophore, to determine what amino acids may participate in the iron binding mechanism and what changes take place in the molecules during oxidation and reduction. An artificial non-heme iron protein which can be prepared from bovine serum albumin has also been used extensively as a model for the iron-binding mechanism.

Methods: Rubredoxin and ferredoxin have been isolated from Clostridium pasteurianum by acetone extraction followed by DEAE chromatography and

447

ammonium sulfate fractionation. Apo-rubredoxin is prepared by precipitation with trichloroacetic and subsequent washing of the precipitated protein with ethanol. Reaction of the cysteine residues of the proteins have been followed both by measuring the formation of the Hg-S bond or the disappearance of the iron chromophore spectrophotometrically. The exchange of exogenous iron with the iron of rubredoxin has been studied using chromatography on Sephadex G-25 to separate the protein from the reactants. The degree of radioactive exchange is expressed as:

$$\frac{\text{Specific Activity of Rubredoxin Iron}}{\text{Specific Activity of Total Iron}} = \text{Percent Equilibration}$$

Optical rotatory dispersion circular dichroism studies were done using a Cary Model 60 spectropolarimeter and the Nuclear Magnetic Resonance studies were done with a Varian Associates 220 megacycle NMR instrument.

Major Findings: Rubredoxin was first isolated from Clostridium pasteurianum in our laboratory in 1965. This protein was shown to have a molecular weight of about 6000, contain 1 mole of non-heme iron, but no inorganic sulfide. This electron carrier protein could be reversibly oxidized and reduced and had a redox potential of about - .057 V at pH 7.0. Rubredoxin is a single electron carrier and it exhibits an electron spin resonance signal at g = 4.3 in the oxidized state but none in the reduced state. Preliminary experiments suggested that the cysteine residues may participate in the iron binding mechanism.

Isolation of Rubredoxin: One of the major problems in the isolation of rubredoxin is the separation of the rubredoxin from ferredoxin. A technique has been developed to quantitatively separate these proteins. Both rubredoxin and ferredoxin are absorbed on DEAE cellulose in 90% saturated ammonium sulfate. The two proteins are eluted from the DEAE cellulose column at lower concentrations of ammonium sulfate. By establishing a reverse gradient (100% saturated $(NH_4)_2SO_4 \rightarrow H_2O$), the two proteins are completely separated on a single column step. Rubredoxin is eluted at about 45% $(NH_4)_2SO_4$ saturation and ferredoxin at about 30% $(NH_4)_2SO_4$ concentration.

Reactivity of the Sulfhydryl Groups: Iodoacetic acid does not appear to react significantly with native rubredoxin or rubredoxin which as been reduced in the presence of 8 M urea. All 4 cysteines of apo-rubredoxin react with iodoacetic in the presence of 0.5 M 2-mercaptoethanol and 8 M urea. The presence of iron in the molecule appears to block reaction of the sulfhydryl groups with iodoacetic acid. Initially it was observed that the mercurial, Na Mersalyl, reacted slowly with the cysteines of native rubredoxin (10% reaction in 24 hrs.). Mercuric acetate however reacts very rapidly (100% in 15 to 30 min). p-Chloromercuribenzoate and p-chloromercurisulfonate react at intermediate rates. The mercurials appear to react with all 4 cysteine sulfhydryls and the destruction of the chromophore appears to occur at the same rate as the formation of the Hg-S bond. These observations strongly suggest participation of sulfhydryls in iron binding.

Fe^{59} Exchange Studies: The iron of rubredoxin exchanges with exogenous iron under certain conditions. The exchange reaction only occurs with ferrous iron, and has a rather sharp pH optimum at 7.0. The exchange also has an absolute requirement for the presence of 2-mercaptoethanol, thus providing further evidence of sulfhydryl participation in iron binding. The exchange reaction is strongly inhibited by iron chelators such as 1, 10-phenanthroline and α,α dipyridyl, and by high concentrations of ascorbic acid. These findings together with the knowledge that rubredoxin exhibits an electron spin resonance signal at g = 4.3 suggest that the iron of rubredoxin is high-spin ferric iron that is ionically held in some manner by the SH groups of the protein. The presence of high-spin ferric iron in rubredoxin has been confirmed by magnetic susceptibility studies.

Nuclear Magnetic Resonance Spectrum of Rubredoxin: Development of the 220 megacycle NMR spectrometer has permitted application of this technique to proteins. It was hoped that by examining the nature of the protons of amino acid residues of rubredoxin some information on the amino acids participating in the chromophore could be obtained. For this purpose about 20 mg of rubredoxin was repeatedly lyophilized and redissolved in deuterium oxide. This material was then examined both in the oxidized and reduced form in the spectrometer. While it is impossible at the present state of knowledge to assign particular resonance bands to particular amino acid protons, the changes and shifts which occurred upon going from the oxidized to the reduced form are very significant. Of particular interest were major changes in the area of the aromatic protons suggesting that some of the aromatic amino acids may participate in the coordination of the iron.

X-ray Crystallography of Rubredoxin: Preliminary X-ray analysis of rubredoxin crystals indicate they belong to the trigonal system with rhombohedral unit cell parameters of $\underline{a}$ = 38.4A and $\alpha = 112^{o}$. The crystals appear to be very suitable for X-ray analysis. It has also been possible to prepare chloroplatinate derivatives using diffusion techniques.

The Artificial Non-Heme Iron Protein Formed From Serum Albumin: Suzuki and Kimura (BBRC 28, 514, 1967) described an artificial non-heme iron protein that could be formed from bovine serum albumin (BSA), iron, and 2-mercapto-ethanol. The final product contained equivalent amounts of iron and inorganic sulfide and had an absorption spectrum similar to ferredoxin. Since these workers added no inorganic sulfide they felt that the labile sulfide of the protein was arising by β elimination of the cysteine sulfur.

In attempting to reproduce this work it soon became apparent that in order to get the artificial non-heme iron protein formed the addition of Na_2S was absolutely essential. By using ^{35}S it was shown that the sulfide was incorporated into the protein, and finally it was demonstrated that the cysteic acid content of BSA and acid denatured, artificial, non-heme iron protein was exactly the same.

6/

It is now clear that the inorganic sulfide does not arise from β-elimination of cysteine in any of the non-heme iron proteins. Since this artificial non-heme iron protein has many properties of the ferredoxin and related compounds it has been further investigated. It is apparent that the role of 2-mercaptoethanol in this reaction is to reduce disulfide bonds and permit the iron to interact with the SH groups. By the use of circular dicroism and preparation of partial derivatives of the artificial non-heme iron protein it appears likely that the iron first interacts with the cysteine sulfhydryls followed by complexion with inorganic sulfide. Further, it would appear that the absorption band at approximately 400 mμ is largely due to specific interaction of the iron with inorganic sulfide.

Significance: The increased knowledge of iron binding by proteins is essential to the understanding of how non-heme iron proteins function in many biological systems. The similarity in many regards between the artificial non-heme iron protein and many biologically active iron proteins makes this compound a useful tool in studying the iron binding mechanism.

Proposed Course of Project: Further studies using physico-chemical techniques are planned to further delineate the active site of rubredoxin and ferredoxin. Rubredoxin in which the iron has been replaced by ^{51}Fe will be examined by the Mössbauer technique. Attempts to reconstitute native rubredoxin from aporubedoxin, after various chemical modifications of the polypeptide chain have been effected, will be made in an effort to obtain more information on other amino acids which may participate in the iron binding mechanism. Further characterization of the artificial non-heme protein will be done to obtain further information on the nature of the chromophore in ferredoxin.

Publications

1. Lovenberg, W. and McCarthy, K.: The origin of inorganic sulfide in artificial non-heme iron protein formed from bovine serum albumin. Biochem. Biophys. Res. Comm. 30: 453-458, Mar. 1968.

652

Serial No. -NHI-238
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Catecholamines and Experimental Hypertension.

Previous Serial Number: None

Principal Investigator: William J. Louis, M.D.

Other Investigators: Sydney Spector, Ph.D., Ryo Tabei, M.D. and Albert Sjoerdsma, M.D., Ph.D.

Man Years

Total: 2.35
Professional: 1.35
Other: 1.0

Project Description:

Objectives: The role of norepinephrine in pathogenesis of experimental hypertension is unclear. However, many workers have reported an increased sensitivity to injected norepinephrine in different forms of hypertension and this suggests that there may be a disturbance in norepinephrine metabolism in hypertension. This view has been strengthened by the recent reports of de Champlain et al (NIMH) that in animals made hypertensive with DOCA and salt there appears to be a disturbance in the binding of norepinephrine which, they presume, leads to an increased leakage of norepinephrine onto the vascular receptors. Recently a strain of animals (SHR) which are genetically hypertensive have become available for study at the N.I.H. It was proposed to look for changes in norepinephrine metabolism in these animals and to compare these changes with those which occur in animals made hypertensive with DOCA and salt.

Major Findings: 1. At all ages studied (5 → 60 weeks) the systolic blood pressure of SHR animals is significantly higher than controls and is usually 170 mm Hg systolic at 15 weeks of age.

2. There is little difference in body weight between SHR animals and controls before 10 → 15 weeks of age at which time individual animals weigh from 200 → 250 gm. Similarly, cardiomegally and renal damage are not apparent in animals weighing less than 250 gm.

3. Studies with tracer doses of tritiated norepinephrine revealed no

difference in the 5 min. accumulation between SHR and control rats suggesting there was no difference in the uptake of norepinephrine. The 24 hour accumulation was much greater in the heart of the SHR than in the Wistar control (168.3 ± 20.3 nc/gm Ht → 54.8 ± 8.9 nc/gm Ht; $p < .001$) suggesting that a diminished rate of release of norepinephrine occurs from the nerve endings of the SHR heart.

4. In spite of the apparent diminished rate of release of ^{3}H-norepinephrine in the SHR the endogenous levels of cardiac norepinephrine were the same in the two groups of animals. This suggests that the diminished rate of release of norepinephrine in the SHR animal is associated with a diminished rate of synthesis of norepinephrine.

5. Studies of the synthesis of norepinephrine from C^{14}-tyrosine confirm the results suggested by the studies using labelled norepinephrine. The C^{14}-studies suggest that the rate of synthesis of norepinephrine from C^{14}-tyrosine is diminished about 40% in the SHR animal and once formed, the norepinephrine is released more slowly in the SHR than in the Wistar control.

Significance: The alterations in norepinephrine metabolism described would, if they occurred throughout the cardiovascular system, tend to diminish the vasoconstrictor effects of endogenous norepinephrine. This may reflect a homeostatic mechanism that comes into play in hypertension and which diminishes the production and release of norepinephrine. These results are also of interest because they contrast with what is found in DOCA-salt hypertension where there appears to be an increased loss of norepinephrine from the nerve endings. The problem with DOCA-salt hypertension is that it is associated with the rapid development of pathological changes, particularly in blood vessels, and it is in these areas of maximal vascular damage that the changes in norepinephrine binding occur.

Proposed Course of Project: It is proposed to study other aspects of norepinephrine metabolism in these animals, notably; urinary excretion of metabolites, monoamine oxidase activity, and levels of fatty acids in the blood. It is also proposed to study older SHR animals with malignant hypertension to see whether with vascular damage the nerve endings are also damaged and now lose norepinephrine which might of itself produce further vascular damage. Such a study might give some insight into the nature of accelerating hypertension.

The results presented do not explain the nature of the hypertension nor the increased vascular reactivity to exogenous norepinephrine. However they do suggest that the latter may be associated with a change in the receptor sensitivity. In view of this it is hoped to study a number of the factors which might well alter norepinephrine receptor sensitivity and attempt to determine the importance of these in the etiology of the hypertension.

Honors and Awards: None

Publications:

1. Louis, W.J., Spector, S., Tabei, R. and Sjoerdsma, A.: Studies on noradrenaline in the heart of the spontaneously hypertensive rat. Lancet In press.

654

Serial No. -NHI-239 (c)
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies Related to Catecholamine Metabolism in Human Disease Processes.

Previous Serial Number: NHI - 164 (c)

Principal Investigator: Karl Engelman, M.D.

Other Investigators: Barry Portnoy, M.D., David Horwitz, M.D., Albert Sjoerdsma, M.D., Ph.D. and Walter Lovenberg, Ph.D.

Man Years

Total: 2.28
Professional: 1.78
Other: .5

Patient Days: 700

Project Description:

Objectives: Sustained investigative interest in catecholamine metabolism and the relationship of catecholamines to altered physiology and disease states has resulted in continued studies in patients with hypertension, migraine headache, and pheochromocytoma. Studies originating in previous years, especially with reference to the potential of inhibition of catecholamine synthesis as a therapeutic maneuver have been continued and expanded. Other projects have dealt with the question of why patients with pheochromocytoma in contrast to other patients with hypertension characteristically have a drop in blood pressure on standing and with continued evaluation of the tyramine test as a pharmacological test for this disease. Efforts in developing a more sensitive method for the accurate determination of catecholamines in plasma samples were extended to fruition, thus enabling studies of plasma catecholamine levels as a reflection of sympathetic nervous activity under various conditions.

Methods: Except for the newly developed double-isotope derivative method for catecholamines, to be described in greater detail under RESULTS, the methodology employed in these studies is similar to that employed in previous years. Biochemical studies have utilized extraction and chromatographic procedures for the isolation of specific compounds coupled with their identification and quantitation by methods employing spectrophotofluorimetric techniques as well as densitometry in both the visual and ultra-violet spectrum. Use of radio-

isotopic techniques including scintillation counting under double isotope conditions has permitted the simultaneous determination of both C^{14} and H^3 labelled compounds. Physiological studies have employed standard electronic techniques for the determination of forearm blood flow, arterial resistance, venous tone and cardiac output in studies related to the orthostatic hypotension for pheochromocytoma. In addition, medical skills have been important in the clinical evaluation, investigative study and therapy of patients with a variety of diseases.

Major Findings: 1. Double-isotope derivative method for catecholamines: Accurate determination in biological samples (plasma, tissue and urine) containing as little as 1 nanogram (10^{-9} gm) norepinephrine or epinephrine is now possible with our newly developed method. The method is based on the conversion of the catecholamines to their C^{14}-methoxy analogues (normetanephrine and metanephrine) in the presence of catechol-O-methyl transferase (COMT) and l-S-adenosyl-methionine-methyl-C^{14} as the methyl donor, and it is so sensitive as to permit the accurate determination of catecholamines in a 5 ml sample of normal plasma. In the case of tissue samples the catecholamines are separated from the solid matter by homogenization in 0.1 N HCl and centrifugation; a portion of the supernatant is taken directly for incubation. A tracer amount of d,l-7-H^3-norepinephrine is added to each sample and provides for an internal recovery. Catecholamines are separated from plasma on a cation-exchange resin (IRC-50) and from urine samples on an alumina column; the eluates from these columns are then prepared for incubation. The incubation-mixture contains a preparation of COMT isolated from rat liver, Mg Cl_2, buffer, S-adenosyl-methionine-methyl-C^{14} (C^{14} AME), ascorbic acid and the sample. After incubation for 1 hour at 37°C the labelled metanephrines formed by the reaction are separated on a cation-exchange column and converted to vanillin with sodium periodate. The vanillin is separated from other radioactive contaminants by a series of extractions and the radioactivity of the product is determined by scintillation spectroscopy in a toluene based phosphor.

The quantity of catecholamine converted to vanillin is based on calculations using the absolute radioactivity (C^{14} dpm) of the vanillin formed and the specific activity of the C^{14} AME. Accurate internal recoveries for each sample are obtained from the H^3 tracer and thus absolute values are obtained for the catecholamine content of each sample. Background counts for the C^{14} blanks range from 35-50 cpm and incubation of standard samples of norepinephrine as small as 1 ng. yield a C^{14} product with radioactivity at least six times background. Recoveries of the tracer norepinephrine through the entire procedure range from 10-50 per cent with the plasma samples having the poorer recovery (10-25 per cent). The method as currently employed measures total catecholamines (norepinephrine plus epinephrine).

The method was initially applied for localization of an ectopic pheochromocytoma which had not been found at previous surgery. Analysis of the catecholamine content of vena caval blood obtained by catheterization localized the tumor venous drainage and permitted successful surgery for the removal of

an ectopic pheochromocytoma situated behind the vena cava and just below the diaphragm. Extension of the method to analysis of venous plasma samples from 21 normal resting subjects has revealed catecholamine concentrations of 0.25 ± 0.07 μg/L (mean ± SD) with a range of 0.13 - 0.35 μg/L. Patients with pheochromocytoma have had peripheral plasma catecholamine concentrations of 2-20 μg/L, and preliminary studies are currently in progress to determine plasma concentrations of catecholamines in a wide variety of clinical conditions as well as following pharmacological and physiological maneuvers.

2. Tyramine Test: Previous preliminary evaluation of the tyramine provocative test for pheochromocytoma has been expanded to include more than one hundred patients. Of the patients with pheochromocytoma who have been studied 20 of 27, (77 per cent) had a systolic pressor response of 20 mm Hg or greater to tyramine injections of up to 1000 μg. This percentage of patients with positive tyramine tests compares favorably with the degree of response to histamine or phentolamine as pharmacological tests for pheochromocytoma, but the absence of morbidity associated with the use of tyramine offers an important advantage. Only 3 per cent of hypertensive patients (3 of 88) studied in a similar manner had false positive responses.

3. Inhibition of Catecholamine Synthesis: Continuing studies with alpha-methyl-para-tyrosine (α-MPT), an inhibitor of the rate-limiting enzymatic step in the synthesis of catecholamines, have been carried out in patients with essential hypertension, pheochromocytoma and migraine headache. Previous results indicating an enhanced antihypertensive effect when α-MPT is administered in combination with other antihypertensive drugs have been confirmed, and studies are being made to determine a possible role of α-MPT for more general use as an adjunct to therapy in patients with resistant hypertension. Additional patients with benign pheochromocytoma were treated with α-MPT as prinicpal preoperative therapy, and others with malignant pheochromocytoma are now receiving this drug chronically. In addition, two patients with migraine headache were studied before and during therapy with α-MPT because of previous suggestion in a patient that it might be useful in this disorder. Despite prolonged placebo and therapy periods it was not possible to confirm our earlier preliminary impressions of a beneficial effect of α-MPT in this disease.

4. Special Studies in Pheochromocytoma: The presence of orthostatic hypotension in patients with pheochromocytoma has remained an enigma. Some have suggested that this may be due to a decreased circulating blood volume, but our previous studies have shown this reduction of blood volume to occur only rarely in these patients. Since we had previously speculated that this defect in blood pressure regulation may be due, instead, to abnormal vasomotor reflexes, we undertook a study in two patients with untreated pheochromocytoma, one with severe and the other mild decreases in blood pressure on standing. Both patients had normal blood volumes measured by both I^{125} albumin and CR^{51} labelled red blood cell dilution studies. Nevertheless, the blood pressure reduction on tilting could be markedly diminished by the rapid

infusion of 1500 ml 5% albumin in saline in the patient who had the more marked orthostatic changes. Physiologic studies of the Valsalva overshoot response, the change in vasomotor tone to a cold pressor test and determination of cardiac output during supine rest and tilt to 60° revealed an absence of normal reflex responses in this patient and an attenuation of responses in the one with only mild orthostatic changes. Since heart rate tended to increase with tilting it appears as though peripheral vasomotor reflexes are abnormal in patients with pheochromocytoma, and this appears to be a reasonable explanation for the orthostatic hypotension.

Proposed Course of Project: Major emphasis during the next year will be directed toward exploiting the availability of a specific and extremely sensitive assay for plasma catecholamine concentrations. It is hoped that by studying the acute changes which may appear in plasma catecholamines during a wide variety of physiological and pharmacological maneuvers, one may get a better index of acute changes in sympathetic nervous activity. Among the interventions planned are the use of various pharmacological adrenergic stimulating and blocking agents and study of the effects of electrical stimulation of the carotid sinus on sympathetic responses to exercise, tilt and other stresses. Comparison of physiological responses in normal and hypertensive patients will also be made in the hope that some differences may be elicited which will permit better understanding of underlying defects in essential hypertension.

Additonal studies are planned with the use of new inhibitors of catecholamine synthesis. Two new drugs, alpha-methyl-phenylalanine and alpha-methyl-5-hydroxy-tryptophan, are anticipated to be ready for first stage clinical testing during this year. The use of the drugs other than α-MPT will simplify the problem of catecholamine determinations in patients receiving these drugs since neither of the two new compounds are metabolized to catechol compounds.

Honors and Awards: None

Publications:

1. Engelman, K., Jequier, E., Udenfriend, S. and Sjoerdsma, A.: Metabolism of α-methyltyrosine in man: Relationship to its potency as an inhibitor of catecholamine biosynthesis. J. Clin. Invest. 47: 568-576, Mar. 1968.
2. Engelman, K., Horwitz, D., Jequier, E. and Sjoerdsma, A.: Biochemical and pharmacological effects of α-methyltyrosine in man. J. Clin. Invest. 47: 577-594, Mar. 1968.
3. Engelman, K., Horwitz, D., Ambrose, I.M. and Sjoerdsma, A.: Further evaluation of the Tyramine Test for pheochromocytoma. New Eng. J. Med. 278: 705-708, Mar. 1968.
4. Engelman, K. and Hammond, W.G.: Adrenaline production by an intrathoracic pheochromocytoma. Lancet. I: 609-611, Mar. 1968.
5. Engelman, K., Portnoy, B. and Lovenberg, W.: Sensitive and specific double isotope-derivative method for the determination of catecholamines in biological specimens. Am. J. Med. Sci. In press.

Serial No. -NHI-240
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Spontaneously Hypertensive Rats (SHR)

Previous Serial No.: None

Principal Investigator: Ryo Tabei, M.D.

Other Investigators: Sydney Spector, Ph. D., William J. Louis, M.D., Sidney Undefriend, Ph. D. (LCB) and Albert Sjoerdsma, M.D., Ph. D.

Cooperating Units: Laboratory of Clinical Biochemistry

Man Years

Total: 1.55
Professional: 1.35
Other: .2

Project Description:

Objectives: There have been various procedures to produce experimental hypertension in animals in order to develop a model which would simulate essential hypertension in man.

In 1963, Okamoto and Aoki reported that they had produced a hypertensive strain of Wistar rats by selective inbreeding. The offsprings of the inbred strain developed hypertension with systolic blood pressure greater than 170 mm Hg at 100 days after birth. The incidence of spontaneous hypertension in 2nd generation of these rats onward was 100%. In this laboratory we have been breeding these animals with the goal of understanding the biochemical lesion which causes the hypertension and secondarily to utilize these rats as a screen for hypotensive agents.

Methods: A colony of SHR animals was established here at the NIH from some breeders brought to the Institute from Japan.

1. Determination of Blood Pressure: For chronological observation and drug studies, the blood pressure was recorded using a tail water plethysmographic method on unanesthetized rats and is reported as systolic pressure. In some instances the blood pressure was recorded directly from the femoral artery under anesthesia.

659

2. Drug Studies: Representative antiadrenergic agents (methyldopa, chlorisondamine, pargyline, phentolamine, chlorothiazide, α-methyl-tyrosine and pronethanol) and some new drugs (4-methoxy -3,5-dihydroxyphenylalanine, 6-OH-dopamine and 5-hydroxy-α-methyl-tryptophan) were administered to the SHR animals intraperitoneally. At varying intervals following drug administration the blood pressure was recorded.

Urinary catecholamine levels in both normotensive and SHR animals were assayed at both room temperature and following cold stress. The enzymatic activities of monoamine oxidase and tyrosine hydroxylase from the heart and brains of both normotensive and SHR animals was compared.

Major Findings: (a) Situation of the SHR colony: The offsprings (500) of all four successive NIH generations developed hypertension with systolic blood pressure greater than 170 mm Hg at 100 days after birth. The level of blood pressure recorded directly was quite comparable to that seen with the indirect plethysmographic method.

(b) Drug effects on blood pressure: Single doses of various hypotensive therapeutic agents, including methyldopa, pargyline, α-methyl-tyrosine, chlorisondamine, chlorothiazide and phentolamine all reduced the systolic blood pressure from 30-50 mm Hg for periods of 2 to 24 hours in hypertensive but not in control animals. The compound 4-methoxy-3,5-dihydroxyphenylalanine depleted endogenous norepinephrine in heart and brain and also lowered the blood pressure of the SHR animal. However, when the β blocker pronethalol was administered to the SHR animals, there occurred a further elevation of the blood pressure. The pressure of the rat rose from a pre-drug level of 180 mm Hg to over 240 mm Hg within one hour following the administration of the drug. The blood pressure remained elevated for 3 to 5 hours. Again, pronethalol exerted little effect on the pressure of the normotensive rat. Another compound tested for blood pressure effects on the SHR animal was 6-OH-dopamine. This agent has been reported to cause degeneration of adrenergic neurons. At a single dose of 20 mg per kg the blood pressure of the SHR animal was reduced 70 mm Hg and the pressure remained at this reduced level for 9 days. Only after 2 weeks did the blood pressure return to its hypertensive level.

(c) The 24 hours urinary excretion of free catecholamines in μg/mg creatinine was similar in SHR and control rats at room temperature. When SHR and normotensive rats were exposed to cold stress (4°C) for 24 hours there occurred a 4 fold elevation in the excretion of free catecholamines in both groups but again there was no significant difference between the two groups.

(d) Enzyme activity: (1) MAO: Heart - 50% increase in SHR compared to controls; brain - no difference between SHR and normotensive. (2) Tyrosine hydroxylase activity in vitro: no changes in adrenals, heart or brain.

Significance: These studies demonstrate the merit of the SHR animals as a model to screen potential hypotensive agents. The changes in MAO activity require further evaluation, particularly in relationship to results of studies on catecholamine metabolism as reported elsewhere by Dr. W. J. Louis.

66

Proposed Course of Project: Investigations will be continued on the mechanism of hypertension in SHR and the effects of various drugs on these animals.

Publications:

1. Spector, S., Tabei, R., Creveling, C.R., Daly, J.W., Witkop, B. and Sjoerdsma, A.: Reduction of tissue norepinephrine and blood pressure by 3,5-dihydroxy-4-methoxy-phenylalanine. Life Sciences, In Press.

Serial Number. NHI-241 (c)
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Monoamine Oxidase (MAO) of Blood

Previous Serial Number: NHI-172 (c)

Principal Investigator: Donald Robinson, M.D.

Other Investigators: Harry R. Keiser, M.D., David Horwtiz, M.D.
Walter Lovenberg, Ph. D. and Albert Sjoerdsma, M.D.,Ph.D.

Man Years

Total: .63
Professional: .63
Other: 0

Patient Days: 350

Project Description

Objectives: Previous studies as part of this project established that blood platelet MAO provides a convenient index of overall body MAO activity in man. The platelet enzyme, similar in many respects to the MAO of liver and brain, provides an easily-obtained measure of the chemical effect of MAO-inhibiting drugs in vivo. α-Ethyl-tryptamine, a drug with weak MAO inhibiting properties in vitro, was withdrawn from clinical use several years ago partly because of the assumed potency of its MAO inhibition in man. Because of the potential therapeutic usefulness of this drug, it was decided to re-evaluate its in vivo effects in man using the platelet assay and other measures of MAO inhibition.

Furthermore, it has been postulated that the blood platelet might serve as a useful model for studying the effects of certain drugs on the sympathetic nerve endings, not only with respect to MAO effect but also to other effects such as uptake and release phenomena. Various guanidine drugs effective in the treatment of hypertension are taken up by and exert a pharmacological effect on the sympathetic neurone. Their action is to inhibit physiologic norepinephrine release resulting from nerve impulse conduction. Certain of this class of drugs, including debrisoquin, chlorobethanidine and bethanidine, are MAO inhibitors of varying potency in vitro. A study of these agents in hypertensive patients was designed to investigate their effect on platelet MAO and tryptamine excretion, as chemical indexes of their MAO-inhibiting properties, for comparison with tyramine and norepinephrine responsiveness, the latter being physiologic measures of the effects of these drugs on the sympathetic nerves.

Methods: The sensitive radioassay for MAO in various tissues, developed as part of this project, was employed in these studies. In most instances, C^{14}-benzylamine was used as substrate because it is most actively deaminated by the enzyme. Platelets were isolated from blood specimens obtained by venipuncture, using a standard technique of differential centrifugation. The platelet-poor plasma remaining after isolation of the platelets was assayed for plasma MAO activity. Urinary tryptamine and tyramine were measured by standard spectrophotofluorometric assays.

Patients were studied during control and treatment periods while on a constant diet. Periodic urine specimens were collected and stored at 4°C for assay of amine excretion. Blood pressure response curves to tyramine and norepinephrine infusion were determined for patients during the control and treatment periods.

Major Findings:

1. dl-Ethyl-tryptamine in daily dosage up to 100 mg was found not to significantly inhibit platelet or plasma MAO in man. This lack of *in vivo* inhibition was confirmed by the failure of the drug to alter tyramine responsiveness, an accepted physiologic test of MAO inhibition. Apparent increases in tryptamine and tyramine excretion during therapy were further investigated and found to be artifactual due to interference by either the drug or its metabolites. In the case of urinary tryptamine, ethyl-tryptamine itself was found to be extracted along with tryptamine during the procedure. Ethyl-tryptamine gives a fluorescence spectrum similar but not identical to tryptamine thereby accounting for the apparent increase in tryptamine excretion. Two urinary metabolites of ethyl-tryptamine, 6-hydroxy-ethyl-tryptamine and a 6-hydroxycarboline derivative, were found to extract along with the tyramine and react also with the nitrosonaphthol. These products also gave fluorescent peaks similar but not identical to the tyramine product, thereby causing a factitious increase in urine tyramine. *In vitro* tests of MAO inhibition by ethyl-tryptamine were also carried out. This drug was found to act as a weak competitive inhibitor of MAO from various tissues including platelet, liver and brain.

It was concluded that ethyl-tryptamine, contrary to previous belief, does not cause significant overall MAO inhibition in man in usual therapeutic dosage. While it is possible that its action is a result of MAO inhibition localized in brain or nervous tissue, it seems probable that its pharmacologic effect is mediated through some other mechanism.

2. A study in man of the effects of the guanidine drugs, bethanidine, chlorobethanidine and debrisoquin and of the related compound, hydralazine, have been completed. Investigation of bretylium and guanethidine is still in progress. None of the drugs were found to significantly inhibit overall body MAO activity. Debrisoquin, however, a known potent MAO inhibitor in certain tissues *in vitro*, was found to inhibit significantly platelet MAO *in vivo*. There were no associated changes in urinary tryptamine excretion however. Hydralazine significantly and irreversibly inhibited plasma MAO

with a doubling of tryptamine excretion during therapy. This increase in urine tryptamine, though probably significant, is not of the magnitude expected in the case of significant overall MAO inhibition.

An interesting alteration in the tyramine and norepinephrine response tests during debrisoquin, bethanidine and chlorobethanidine administration was noted. In each case, a significant but moderate increase in sensitivity to both tyramine and norepinephrine infusion was demonstrated during the period of drug administration. The increase in tyramine responsiveness was not of the magnitude observed with potent MAO inhibitors nor was there the prolongation of the tyramine pressor effect seen with other MAO-inhibiting drugs. The increase in norepinephrine sensitivity, which paralleled that of tyramine, also is not characteristic of the effect of MAO inhibition. Therefore, it is likely that the guanidine drugs exert an effect on the sympathetic membrane involving at least norepinephrine uptake, which may relate to their antihypertensive action, rather than through the mechanism of MAO inhibition. Studies are in progress to investigate further their mechanisms of action.

Significance: 1. The platelet MAO assay was utilized in part to establish that the antidepressant drug, ethyl-tryptamine, does not cause significant MAO inhibition in man.

2. The mechanism of action of the guanidine class of antihypertensive agents has been further investigated in vivo by means of the platelet MAO assay, urine amine excretion, and tyramine and norepinephrine response testing. Although studies are still in progress, it would appear that the action of these drugs on sympathetic nerves is not mediated through MAO inhibition.

Proposed Course of Project: The study of the in vivo effects of other drugs of the guanidine class of antihypertensive agents will be continued utilizing the platelet assay as well as urine amine excretion and the tyramine response test.

Honors and Awards: None

Publications:

1. Robinson, D.S., Lovenberg, W., Keiser, H. and Sjoerdsma, A.: Effects of drugs on human blood platelet and plasma amine oxidase activity in vitro and in vivo. Biochem. Pharmacol., 17, 109-119, Jan. 1968.

Serial No. -NHI-242 (c)
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Clinical Investigation of Cardiovascular Drugs

Previous Serial Number: NHI-163

Principal Investigator: David Horwitz, M.D.

Other Investigators: Karl Engelman, M.D., Donald Robinson, M.D. and Albert Sjoerdsma, M.D., Ph.D.

Man Years

Total: 1.55
Professional: 1.2
Other: 2.0

Patient Days: 2100

Project Description:

1. Factors Altering Cardiovascular Responses to Tyramine and Norepinephrine.

Objectives: Cardiovascular responsiveness to infusions of the amine, tyramine, may be used to acquire information in patients about norepinephrine storage, activity of the enzyme monoamine oxidase, and the efficiency of amine uptake mechanisms in sympathetic nerves as follows: 1) Since tyramine acts indirectly by causing the release of the sympathetic mediator norepinephrine, its pressor potency is reduced in the presence of pronounced depletion of norepinephrine stores and is restored with repletion of norepinephrine, 2) Tyramine is totally dependent on the enzyme monoamine oxidase (MAO) for degradation and consequent termination of its action; in the presence of a drug with potent MAO inhibitory activity the pressor effects of tyramine are dramatically potentiated and prolonged but effects on norepinephrine are minor, and 3) Drugs inhibiting the uptake of amines into sympathetic nerves depress the pressor potency of tyramine but yield potentiation of norepinephrine itself since the latter is partially dependent on uptake by nerve cells for termination of its action.

Our recent investigations utilizing tyramine have been concerned with the central nervous system stimulant, α-ethyl-tryptamine (etryptamine) and with hypotensive agents which have included debrisoquin and the benzylguanidines, bethanidine and an analogue, 392 C 60. Initial emphasis was

placed on an assessment of the role of MAO inhibition in the action of these agents. In the case of etryptamine, speculation about MAO inhibitory activity was prompted by resemblances of central stimulatory effects to those produced by various known MAO inhibitors and by striking increases in levels of fluorophores resembling urinary tyramine and tryptamine which were induced by the drug. Interest in a potential role of MAO inhibition in the pharmacologic actions of the benzylguanidines derived from knowledge of the demonstrated weak overall MAO inhibitory effects in vivo and in vitro in animals and from singular localization of these drugs in sympathetic nerves so that a disproportionate local MAO inhibitory effect was plausible; in addition, hypotensive properties have been found in a wide variety of potent MAO inhibitors previously studied in this Branch.

Methods: Observations were made in subjects with mild to moderate hypertension who received conventional doses of the drugs. Concurrent determinations of effects on MAO as revealed in assays of plasma and platelet MAO and in urinary levels of tyramine and tryptamine were performed. Dose-response curves relating increments of systolic blood pressure to different infusion rates of tyramine and norepinephrine were prepared during control and treatment periods.

Major Findings and Significance: D and/or L isomers of etryptamine were administered to four subjects in doses of 100 mg per day for approximately a week; they produced no increase in pressor responsiveness to infused tyramine and caused no significant changes in levels of plasma and platelet monoamine oxidase (biochemical observations are discussed in detail elsewhere by Dr. Robinson). There was thus no evidence of MAO inhibition during administration of therapeutic doses of etryptamine.

Debrisoquin, bethanidine, and 392 C 60 administered for at least a week in doses reducing blood pressure caused three-to-ten fold increases in pressor responsiveness to tyramine; the duration of pressor effects was not significantly increased, however, as is usual with MAO inhibition, and increases in responsiveness to tyramine were closely paralleled by increases in responsiveness to the pressor effects of norepinephrine. It was concluded that effects differed from those of known potent monoamine oxidase inhibitors; this was similar to conclusions derived from biochemical observations which are discussed elsewhere. The effects on responsiveness to tyramine and norepinephrine differed from those of known norepinephrine-depleting agents such as reserpine and from agents such as cocaine and imipramine which are considered to inhibit amine uptake at the neuronal membrane. This phenomenon is under continuing study.

2. Responsiveness to Amines in Patients with Pheochromocytoma.

Objectives: The enhanced pressor responsiveness of patients with pheochromocytoma to tyramine was demonstrated in this section and has provided the basis for a useful pharmacologic screening test for this disorder. In an effort to explain this phenomenon studies of the responsiveness of the forearm circulation to intra-arterial infusions of tyramine and norepinephrine were performed in patients with pheochromocytoma and essential hypertension.

Methods: Changes in forearm blood flow were measured by mercury-in-rubber strain gauge plethysmography during graded 5 minute intra-arterial infusions of the amines.

Major Findings: Studies have been completed in five patients with pheochromocytoma and two hypertensive subjects. Preliminary findings suggest an enhanced sensitivity to tyramine associated with decreased sensitivity to norepinephrine in the forearm circulation of patients with pheochromocytoma who have positive pharmacologic tests with tyramine.

3. Effects of Alpha-Methyl-Dopamine.

Objectives: The hypotensive agent methyldopa is considered to work through conversion to the amine metabolites, α-methyl-dopamine and α-methyl-norepinephrine but it is not known whether predominant effects are exerted in the central nervous system or in peripheral sympathetic nerves. It was therefore of interest to establish whether the metabolite, α-methyl-dopamine, was a useful hypotensive agent and whether its different distribution between central nervous system and peripheral nerves yielded a different spectrum of responses.

Methods: Alpha-methyl-dopamine was administered to two hypertensive subjects for one week in divided oral doses of 200 mg/day. Fluorometric assays for urinary α-methyl-dopamine were performed after passage of samples over an alumina column and oxidation of eluates with potassium ferricyanide.

Major Findings and Interpretation: Oral α-methyl-dopamine was devoid of symptomatic or cardiovascular effects with single doses of up to 125 mg and sustained daily divided doses of 200 mg in the two subjects tested. Assays of the urine revealed that approximately one third of the administered dose could be detected in the urine but that virtually all of the drug was present in a conjugated form; the latter was considered to be a possible explanation for the lack of observed activity of the drug.

4. Use of Alpha-methyl-para-tyrosine (α-MPT) in Migraine.

Objectives: α-MPT is a potent inhibitor of norepinephrine synthesis with associated sedative and mild hypotensive effects in man. Initial observations in one patient were suggestive of useful effects on migraine and observations were therefore pursued in an additional two hospitalized patients with severe migraine.

Major Findings: Results of treatment with α-MPT were suggestive of useful effects in comparison with placebo therapy but the drug appeared to be of limited potency and observations were not considered to be conclusive.

5. Plasma Corticosterone Levels in Hypertension.

Objectives and Findings: Subjects with abnormally low plasma renin levels and with poor responsiveness of renin levels to salt restriction, postural changes and administration of diazoxide have been reported among the so-

called "essential" hypertensives. It was considered possible that some subjects with hypertension of unexplained cause might have incomplete defects in cortisol synthesis with accumulation of precursor steroids and resultant hypertension. In consequence a group of hypertensives characterized by a strong family history or early age of onset were screened for increased plasma levels of corticosterone. Twenty-one subjects have been investigated with negative findings to date.

Proposed Course of Project:

1. Studies of drug effects on responses to tyramine and norepinephrine will be pursued.
2. Plethysmographic observations of responses to norepinephrine and angiotensin will be made in normals and subjects with hypertension.
3. Further "steroid screening" of patients with hypertension will be pursued.

Publications: None

Serial No. -NHI-243 (c)
1. Experimental Therapeutic Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Metabolism of Hydroxyproline and Collagen

Previous Serial Number: NHI- 165 (c)

Principal Investigator: Harry R. Keiser, M.D.

Other Investigators: Albert Sjoerdsma, M.D., Ph.D., Donald Robinson, M.D., Robert I. Henkin, M.D., Ph.D., Morley Kare, Ph.D.

Cooperating Units: Endocrinology Branch, NHI
Physiology Department, North Carolina State University

Man Years

Total: 3.2
Professional: 1.2
Other: 2.0

Patient Days: 1500

Project Description:

Objectives: 1. The development of methods for studying collagen metabolism in man in animals. 2. The application of these methods to the study of collagen metabolism in normal man and to the study of the pathogenesis of various collagen diseases. 3. The selection and evaluation of possible therapeutic agents in the treatment of collagen disorders.

Methods: Hydroxyproline (HOPr) because of its uniqueness in collagen is a useful tool in studying collagen metabolism. The methodology for measuring HOPr in urine and plasma was developed and tested in this laboratory. A "collagen profile" applied to tissues involves analysis of minced specimens of dermis obtained by punch biopsy or surgery for water content, total collagen content per unit of dry weight and per unit of surface area, percent of collagen extractable into neutral salt or 0.5 M acetic acid and the degree of intra-molecular cross-linking of acid-soluble collagen. We also study several enzymes involved in collagen synthesis. Proline hydroxyiase activity in dermis is measured by a modification of the methods developed by Drs. Hutton and Udenfriend in LCB. Monoamine oxdiase (MAO) activity is measured in skin minces using radioactive benzylamine as substrate. Recently we have developed a method for determining the rate of collagen synthesis in man by measuring the rate of proline incorporation into dermal collagen in vitro.

Screening tests of drugs for effects on collagen metabolism in rats were performed for 14 days with the drug either injected I.P. daily or given mixed with food. The studies of the effects of D-Penicillamine (D-Pen) on rats were started in North Carolina by Dr. Kare who prepared and taste-tested the animals. Forty male weanling Holtzman rats were divided into groups of 8 animals each and fed a diet of ground purina chow containing 15% dextrose. One group served as controls, another group received D-Pen (10 gm/kg diet) for 28 days and the other three groups received D-Pen for 52 days. The last two groups were given copper in either a low dose (50 mg/kg diet) or a high dose (10 gm/kg diet) for the last 24 days. The animals were brought to the N.I.H. On the 52nd day 24 hour urine collections were made and the animals were then killed and indices of collagen metabolism were ascertained in all groups.

Major Findings: We have now administered D-Pen to 4 patients with scleroderma in doses of 2-3 gms. daily for over one year. While there have been definite increases in the percentage of acid extractable collagen in the dermis of these patients these changes have not been of the same magnitude observed in patients with normal skin treated with D-Pen, i.e. cystinuria and Wilson's disease. There have been no objective changes noted in any of the other measurements we have made, including range of joint motion, pulmonary function, esophageal motility, or body weight. The side-effects of D-Pen have been many and varied, and have included allergic reactions, anemia, leukopenia and thrombocytopena, alone or in combination, loss of taste, and severe anorexia and malaise. The most interesting effect was the decrease in taste acuity for sweet and salt. This has now been found during treatment with D-Pen in 4 (4%) of 100 patients with Wilson's disease and in 23 (32%) of 73 patients with other diseases. The loss of taste in non-Wilson's disease patients was associated with low levels of serum copper and ceruloplasmin which indicate copper deficiency except in patients with Wilson's disease (Sternlieb and Janowitz, J.C.I., 43:1049, 1964). In patients with Wilson's disease serum levels of copper and ceruloplasmin are low while tissue copper levels are markedly elevated and can only rarely be reduced to normal after even prolonged treatment with D-Penicillamine. Thus the low incidence of taste loss in patients with Wilson's disease treated with D-Pen would seem to be related to their high levels of tissue copper. When copper is administered to non-Wilson's disease patients taking D-Pen who have decreased taste acuity, plasma copper and ceruloplasmin levels return to normal range within 3 to 5 days while taste acuity doesn't return to normal for 2½ to 3 weeks.

The rate of proline incorporation into human derman collagen was found to be rapid (10-70 dpm/μM tissue proline) in young growing subjects, and much slower (4-6 dpm/μM) in adults. The rate was increased in one adult with active acromegaly to a level (13 dpm/μM) comparable to that found in growing children. There was no apparent difference between obviously involved skin and apparently uninvolved skin in patients with scleroderma and between normals and patients with scleroderma.

D-Pen has been given to rats in a series of studies to examine these effects in greater detail. D-Pen treated animals became "sickly", neuro-

logically damaged, had loss of taste for salt as measured by preferences for either .15 M or .30 M salt over distilled water, were anemic, leukopenic and thrombocytopenic and had collagen abnormalities in 100% of the treated animals. The collagen abnormalities will be the only ones discussed here. They consisted of classic lathyritic changes. The animals were about ½ the size of normals, had scruffy-looking coats, and marked kyphoscoliosis. X-rays revealed deformed bones, some exostoses and subperiosteal new bone formation. Urinary creatinine excretion was ½ of that of normals, while urinary HOPr was only mildly reduced; thus urinary HOPr/mg creatinine was nearly double that of normal animals. In the collagen profile of D-Pen-treated animals, in comparison to controls, the percent of water content and total collagen content expressed per unit weight of dermis was not significantly altered, but collagen content expressed in terms of unit of surface area was reduced by 1/3. The percentage of neutral salt soluble collagen was increased dramatically, being 7.2 ± 1.3 vs. 0.5 ± 0.1% for controls. D-Pen-treated animals also had significantly lower levels of proline hydroxylase activity (85 ± 22 vs. 274 ± 20 dpm/mg dermis/hr.) and of MAO activity (0.47 ± .04 vs. 0.83 ± .05 mμM/mg dermis/hr.). Low doses (0.6 mg/kg/day) of copper partially corrected and high doses (30 mg/kg/day) significantly or completely corrected all of D-Pen induced abnormalities when the copper was added to the animal's diet while D-Pen administration was continued. The collagen abnormalities were not prevented by giving pyridoxine. Pair-fed control and pyridoxine-deficient animals studied in a second set of experiments were the same as control animals.

A total of 21 different drugs were tested in our screening program. Only 3 agents, β-aminopropionitrile (BAPN), D-Pen, and isoniazid (INH) had lathyritic properties as indicated by increased urine HOPr and increased neutral salt soluble collagen content of skin. Only INH inhibited skin MAO and none of these agents had any effect on liver MAO. A number of classic MAO inhibitors inhibited both skin and liver MAO but had no apparent effect on dermal collagen.

Significance: D-Pen is not useful alone in the treatment of patients with scleroderma. The finding that copper repletion returns taste sensitivity to normal indicates that copper is involved in the mechanisms of taste. The finding that copper prevents the collagen abnormalities (as well as the anemia and neurologic abnormalities) of D-Pen administration indicates that copper is involved in collagen metabolism. It seems possible that copper may be useful in reducing some of the toxicity of D-Pen when the drug is administered for treatment of cystinuria and rheumatoid arthritis of man.

Direct measurements of collagen synthesis rates in man have never been done before. The observed rates correlate well with observed growth in children and in adults with active acromegaly. This should provide a useful tool to study collagen metabolism in man.

The use of measurements of skin and liver MAO activities has not been helpful to date in the selection of potentially useful drugs for the treat-

ment of collagen disorders. It has provided some insight into collagen metabolism but has confirmed our feeling that the MAO activity we measure in minced dermis probably does not represent the specific enzyme involved in cross-linking of collagen and elastin.

Proposed Course of Project: 1. We plan further studies of the effects of copper, copper-chelators, and drugs with free sulfhydryl groups on the metabolism of collagen in animals.

2. We also plan to study other heavy metals for their effects on collagen metabolism.

3. We will study the various enzymes involved in collagen synthesis in various disease states in order to learn more about their pathogenesis.

Honors and Awards: None

Publications:

1. Keiser, H.R., Harris, E.D. and Sjoerdsma, A.: Studies on beta-aminopropionitrile in animals. Clin. Pharmacol. & Therap. 8: 587-592, July-August 1967.

2. Keiser, H.R. and Sjoerdsma, A.: Studies on beta-aminopropionitrile in patients with scleroderma. Clin. Pharmacol. & Therap. 8: 593-602, July-August 1967.

3. Keiser, H.R., Henkin, R.I., Bartter, F.C. and Sjoerdsma, A.: Loss of taste during therapy with Penicillamine. J.A.M.A. 203: 381-383, Feb. 1968.

4. Henkin, R.I., Keiser, H.R., Jaffe, I.A., Sternlieb, I. and Scheinberg, I.H.: Decreased taste sensitivity after D-Penicillamine reversed by copper administration. Lancet II: 1268-1271, Dec. 1967.

Serial No. -NHI-244
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Monoamine Oxidase (MAO) of Skin

Previous Serial Number: NHI 172 (c)

Principal Investigator: Donald Robinson, M.D.

Other Investigators: Harry R. Keiser, M.D., David Horwitz, M.D.
Walter Lovenberg, Ph. D. and Albert Sjoerdsma, M.D.,Ph.D.

Man Years

Total: .22
Professional: .22
Other: 0

Project Description

Objectives: The cross-linking mechanism in both collagen and elastin is presumably initiated by oxidation of specific epsilon amino groups of polypeptide lysine to the corresponding aldehydes which then undergo aldol condensation reactions. Using radioactive amines as precursors, in a method previously reported from this laboratory, amine oxidase activity was demonstrable in skin from man and rat. Our objective was to compare skin amine oxidase with that of liver with respect to enzyme kinetics and substrate and inhibitor specificity.

Methods: Initial studies and the comparison of amine oxidase activity in human skin and rat skin were done with minces of skin as the enzyme source. The substrate kinetics and inhibitor studies of rat skin and liver amine oxidase were done on partially purified enzymes. The rat skin amine oxidase was prepared from finely sliced dermis, pulverized in liquid nitrogen, and digested with bacterial collagenase. Upon subsequent homogenization and centrifugation the major activity was in the supernatant fraction and was precipitated by 55% saturation with ammonium sulfate. The enzyme at this stage was insoluble, but fully active and about tenfold purified over the crude tissue homogenate. No attempts were made at further purification. The rat liver amine oxidase was prepared from homogenates of whole liver by a modification of the method of Nara et al (J.B.C. 241, 2774, 1966).

Major Findings: Minces of human and rat skin catalyze the oxidation of benzylamine to benzaldehyde at the rate of 1-2 μM/g skin/hr. This is less than that found in rat liver homogenates, 33 μM/g liver/hr, but significant and readily measurable. The partially purified amine oxidases from rat skin and rat liver were tested for their relative rates of oxidation of

benzylamine, tryptamine, tyramine, serotonin, kynuramine and β-aminopropionitrile (BAPN). The Michaelis constants and relative oxidation rates of the substrates were similar for both enzyme sources with benzylamine being the most active substrate tested. The most striking difference noted was that the Michaelis constants with benzylamine were 2×10^{-5} M for rat skin and 5×10^{-4}M for rat liver amine oxidase. There was no demonstrable diamine oxidase activity in rat skin uring cadaverine as substrate. The rat skin enzyme is inhibited by the classic inhibitors of rat liver amine oxidase i.e. JB-516, tranylcypromine, and iproniazid, at concentrations similar to those effective for the latter amine oxidase at a concentration 1/600 of that required for equal inhibition of skin amine oxidase. Penicillamine and BAPN were not significant inhibitors of skin amine oxidase in vitro. Human skin amine oxidase is inhibited by the same inhibitors that inhibit the enzyme from rat skin and to about the same extent. Tris buffer inhibited the skin enzyme while exerting no effect on the liver enzyme. Preliminary in vivo studies on animals made lathyritic with BAPN failed to reveal any difference in skin amine oxidase activity when compared with normal animals.

Significance: These studies indicate that there is significant amine oxidase activity in human and rat skin. The amine oxidase activity in rat skin and human skin are similar with respect to the inhibitors studied. While the amine oxidase activity in rat skin is similar in many ways to that in rat liver homogenates there are also distinct differences suggesting that these represent either different enzymes or iso-enzymes. The failure of BAPN and Penicillamine to inhibit skin amine oxidase in vitro and in vivo raises questions as to the role of the amine oxidase to the cross-linking mechanism.

Proposed Course of Project: Further studies will be directed along two lines: 1. Attempts to isolate, identify and study the specific amine oxidase involved in the cross linking of collagen and elastin and, 2. Assay of skin amine oxidase activity in scleroderma and other disease states in search of a possible correlation with pathologic processes.

Honors and Awards: None

Publications:

1. Lovenberg, W., Dixon, E., Keiser, H.R. and Sjoerdsma, A.: A comparison of amine oxidase activity in human skin, rat skin and rat liver: relevance to collagen cross-linking. Biochem. Pharm. In Press.

674

Serial No. -NHI-245

1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Regulation of Norepinephrine Biosynthesis

Previous Serial No.: NHI 170

Principal Investigator: Sydney Spector, Ph. D.

Other Investigators: Robert Gordon, Ph.D., Wallace Dairman, Ph. D. (LCB), Albert Sjoerdsma, M.D., Ph.D. and Sidney Udenfriend, Ph. D., (LCB).

Cooperating Unit: Laboratory of Clinical Biochemistry

Man Years

Total: .85
Professional: .45
Other: .40

Project Description:

Objectives: The rate of synthesis and release of norepinephrine in sympathetically innervated tissues varies with the degree of nerve stimulation, and within the nerve exists a mechanism for the regulation of norepinephrine synthesis. In previous reports we have shown that if the endogenous levels of tissue norepinephrine are reduced by nerve stimulation there occurs an acceleration of norepinephrine biosynthesis; conversely we also showed that following an accumulation of endogenous norepinephrine content after the administration of a monoamine oxidase inhibitor, the rate of synthesis is reduced.

Studies were undertaken to determine whether administration of adrenergic-blocking agents modifies the rate of synthesis of the neurotransmitter substance and whether the site of regulation is at the rate limiting step.

Methods: Rats were pretreated with phentolamine (5 mg/kg) and phenoxybenzamine (25 mg/kg), doses which effectively block the α-adrenergic receptors. A pulse label of tyrosine-^{14}C or dopa-^{3}H was then administered intravenously to the rats to study rates of incorporation of isotope into norepinephrine. The effects of the α-adrenergic blocking agents were tested *in vitro* on tyrosine hydroxylase purified according to the method of Nagatsu *et al*. Tyrosine hydroxylase activity was also assayed in the adrenal glands of rats which had been treated with the α-adrenergic blockers.

Major Findings: Both phentolamine and phenoxybenzamine caused a 5 fold acceleration of norepinephrine and epinephrine synthesis from tyrosine-^{14}C. Correlation between the α-blockade and the effects on the incorporation from tyrosine-^{14}C into catecholamine was also demonstrated. When receptor blockade was maximal, synthesis was increased maximally and when after a period of time blockade had worn off, incorporation of label from tyrosine had fallen. The increased radioactive incorporation was observed only with tyrosine as the precursor. This indicates that the effect of the α-adrenergic blockers is at the tyrosine hydroxylase step.

A direct effect of the α-blockers on the enzyme tyrosine hydroxylase was ruled out since no effect could be detected on the in vitro activity of adrenal tyrosine hydroxylase.

Significance: The decreased receptor response brought about following α-blocking agents appears to result in a stimulation of adrenergic nerve activity to maintain homeostasis through possibly some reflex mechanism. These drugs not only stimulate catecholamine synthesis in peripheral tissues but also in the brain.

Proposed Course of Project: Investigations will be continued on the controlling mechanisms for catecholamine biosynthesis and the effects of various drugs on this system.

Publications

1. Spector, S., Gordon, R., Sjoerdsma, A. and Udenfriend, S.: End-product inhibition of tyrosine hydroxylase as a possible mechanism for regulation of norepinephrine synthesis. Molec. Pharm. 3: 549-555, Nov. 1967.

676

Serial No. -NHI-246
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Steroidogenesis: Mechanisms and Control in Hypertension

Previous Serial Number: None

Principal Investigator: William C. Govier, M.D., Ph.D.

Other Investigators: Walter M. Lovenberg, Ph.D., Albert Sjoerdsma, M.D., Ph.D.

Man Years

Total: .53
Professional: .53
Other: 0

Project Description:

Objectives: At the present time, antihypertensive chemotherapy is directed primarily toward altering the production, release or response to catecholamines. A wide variety of compounds is available for this purpose. It is recognized, however, that hypertension is a multifaceted problem and that catecholamines are not involved in all cases. The adrenal cortex is a second location at which dysfunction may lead to hypertension. It is known, for example, that the mineralocorticoid deoxycorticosterone, when combined with a salt load, can lead to hypertension in experimental animals. This project will examine adrenal steroid production using three different approaches. First, the mechanism by which ACTH initiates steroid production through the adenyl cyclase 3',5' cyclic AMP-desmolase chain of events will be examined. Second, the individual enzymatic reactions involved in the conversion of cholesterol to active steroids will be examined with regard to their necessary cofactors and possible inhibitors. Third, particular attention will be paid to two cofactors which are apparently essential at all steps of cholesterol conversion - adrenodoxin and TPNH. The primary objective of these studies is to obtain additional basic information regarding steroidogenesis which may ultimately result in the ability to exercise some control over steroid production and steroid effects and thereby affect one group of hypertension cases.

Methods: A current theory states that ACTH initiates steroidogenesis through a mechanism involving protein synthesis. Protein synthesis will be studied by measuring incorporation of ^{14}C-amino acid into rat adrenal slices incubated with and without ACTH. The crude protein of the adrenal will be separated

into several fractions by ammonium sulfate precipitation in order to determine whether stimulation of synthesis of a particular protein can be demonstrated.

The conversion of cholesterol to pregnenolone by rat adrenal, the rate-limiting step in steroid production, will be studied with ^{14}C-cholesterol The conversion can be followed by extracting the steroids from the incubation medium with chloroform, chromatographing the material on a thin-layer plate and counting the resulting ^{14}C-pregnenolone.

Adrenal deoxycorticosterone and its conversion to corticosterone will be measured in the rat. Incubation of deoxycorticosterone with adrenal homogenates or extracts of adrenal mitochondria in the presence of TPNH results in the formation of corticosterone. This steroid can be extracted from the medium with chloroform and measured fluorimetrically by treatment with H_2SO_4 in ethanolic solution. The starting compound, deoxycorticosterone, does not fluoresce under these conditions.

The characterisitics of adrenodoxin, a mammalian non-heme iron protein electron carrier which is essential for steroidogenesis, will be examined immunologically and compared to similar proteins obtained from non-mammalian sources. Adrenodoxin will be isolated from pig adrenals and injected into rabbits in order to produce antibodies. The antigen-antibody reaction can then be used to determine whether adrenodoxin is identical to other non-heme iron proteins and to localize the protein in various tissues.

The TPNH concentration in rat adrenals will be measured by a standard fluorimetric method.

Major Findings: This project has just been initiated and time has been spent setting up the necessary assay systems and obtaining a working supply of adrenodoxin. The procedures for adrenodoxin isolation, for measuring cholesterol conversion to pregnenolone, for measuring amino acid incorporation into protein and for measuring the conversion of deoxycorticosterone to corticosterone are now in working order. Study of amino acid incorporation into adrenal protein has begun.

Significance: It is too soon to comment upon the significance of results so far. The possible ultimate significance of the project has been considered above.

Proposed Course of Project: Advantage will be taken of the availability of a strain of rats which exhibit spontaneous hypertension. The results of the various experimental procedures will be compared in normal, spontaneously hypertensive and hypophysectomized rats. First priority will be given to a study of possible protein synthesis under the influence of ACTH and the relationship of ACTH induced steroidogenesis to the steroidogenesis produced by TPNH. The available evidence linking protein synthesis to ACTH effect is

circumstantial and rests on the ability of known protein synthesis inhibitors to prevent ACTH induced steroidogenesis. It is possible, however, that this effect is due to a separate action of the inhibitors. A somewhat disturbing note in the current hypothesis is that the steroidogenesis induced by TPNH, a necessary cofactor for all steroid production, is unaffected by protein synthesis inhibitors. This may imply that more than one steroidogenic pathway exists and that protein synthesis may not be a requirement. Studies of possible TPNH production under ACTH stimulation will be done in an attempt to approach this problem. It is apparent that proper control experiments have not been performed in most of the available literature concerning the protein synthesis problem. A complete reevaluation of this probelm could be in order.

As the assays for various steroid conversion steps become operative and the effects of compounds inhibiting these steps become clear, it is hoped to be able to initiate a program in suitable patients of adrenal cortical suppression and selective replacement in order to approach the problem of the role of certain steroids in the pathogenesis of some forms of hypertension.

Honors and Awards: None

Publications: None

Serial No. -NHI-247
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A Study of the Factors which Influence the Activity of Liver Alcohol Dehydrogenase (Inhibition by Thyroxine and Related Compounds).

Previous Serial Number: NHI - 171

Principal Investigator: Walter Lovenberg, Ph.D.

Other Investigators: None

Man Years

Total: .58
Professional: .08
Other: .5

Project Description:

Objectives: Alcohol dehydrogenase in mammals catalyzes, in addition to the oxidation of alcohols, the interconversion of Vitamin A and retinine and the formation of alcohols as metabolites of various amines. Since it was previously shown in this laboratory that thyroxine and triiodothyronine (T_3) are potent inhibitors of horse liver alcohol dehydrogenase (LADH) it was of interest to know how these compounds interact with this enzyme and of what relevance this is to other dehydrogenase enzymes.

Methods: Alcohol dehydrogenase activity was assayed by measuring changes in reduced pyridine nucleotide by the typical spectrophotometric method. Optical rotatory dispersion measurements were done using a Cary Model 60 Spectropolarimeter.

Major Findings: The majority of the work on this project was completed in the previous year and the results are summarized below. Thyroxine and T_3 were uncompetitive inhibitors with respect to either the substrate or cofactor causing significant inhibition in concentrations as low as 10^{-6} M. The inhibition was pH dependent with the inhibitors being most effective when their phenolic groups were unionized. The inhibition by these compounds was not reversed by zinc ions and was apparently not due to complexine with the zinc of the enzymes. Thyroxine and T_3 apparently did not cause dissociation of the enzyme into subunits as in the case with glutamic dehydrogenase. Equilibrium

680

dialysis experiments also indicated that the thyroid hormones had no effect on the overall ability of the apoenzyme to bind the coenzyme, NADH. Subsequent studies however indicated that the binding of the coenzyme was altered. Thyroxine and T_3 caused a marked quenching of the enhanced fluorescence that occurs when NADH is bound to the enzyme. The Cotton effect in the optical rotatory dispersion spectrum which is attributed to the bound pyridine nucleotide is also greatly diminished in the presence of T_3. There appears however to be no measurable change in the overall helicity of the protein as determined by Moffitt-Yang plots. 1,10-Phenanthroline is known to complex with the enzyme bound zinc of LADH and yield an optically active chromophore. The optical rotatory dispersion spectrum of the chromophore was slightly altered by the presence of T_3 suggesting that T_3 may be bound near the zinc site of the enzyme.

Consideration of these findings in light of Theorell's 2-step coenzyme binding mechanism suggests a possible mechanism for the thyroid-inhibition. Normally the adenosine diphosphate ribose portion of the NADH first binds to the enzyme which results in a conformational change in the protein allowing the nicotinamide portion of the coenzyme to bind at or near the zinc site of the enzyme. This causes the formation of a catalytically active holoenzyme which shows enhanced fluorescence. It is possible that the thyroid hormones interfere with the second step of the binding causing a quenching of fluorescence and a loss of enzymatic activity.

Significance: Since thyroxine and related compounds inhibit many other pyridine nucleotide-linked dehydrogenases this study provides an excellent example of how thyroid hormones can interact with a target protein molecule and exhibit a regulatory effect on an enzyme.

Proposed Course of Project: No further experimentation is planned for this project.

Honors and Awards: None

Publications:

1. McCarthy, K., Lovenberg, W. and Sjoerdsma, A.: The mechanism of inhibition of horse liver alcohol dehydrogenase by thyroxine and related compounds. J. Biol. Chem. In press.

Serial No. -NHI-248 (c)
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Metabolism of Bradykinin

Previous Serial Number: None

Principal Investigator: Harry R. Keiser, M.D.

Other Investigators: Albert Sjoerdsma, M.D., Ph.D., John Pisano, Ph.D. (LCB)

Cooperating Units: Laboratory of Clinical Biochemistry

Man Years

Patient Days: 10

Total: .06
Professional: .06
Other: 0

Project Description:

Objectives: Bradykinin, a potent vasoactive peptide found in man and animals, has been implicated in a number of physiologic and pathologic processes. The enzymes and substrates involved in the synthesis of bradykinin have been studied extensively. While the enzymes which catabolize bradykinin have also been studied, the actual metabolic products, which are physiologically inactive have not been identified. Bradykinin labelled with Proline-C^{14} (U.L.) in the second position was synthesized recently for us by New England Nuclear Corporation. Our objectives, using this material, were to identify the urine metabolites of bradykinin in man, determine if they were unique to bradykinin, and then study the kinetics of bradykinin metabolism in normal man and in patients.

Methods: In initial studies small doses of bradykinin were given either intravenously or intraperitoneally to mice, rats and dogs to determine the disposition of radioactivity. This data was necessary to calculate dosages and to obtain clearances for administration to patients. Two patients with carcinoid syndrome were given intravenous infusions of 10 μC of radioactive bradykinin, specific activity 101 μg/μC, over a period of 25 to 60 minutes. Fractional urines were collected, frozen and later assayed for total radioactivity. The collections from the first 1 hour were desalted, chromatographed on Beckman PA-27 resin, and fractions were collected and examined for total radioactivity, amino acid content after acid hydrolysis and on Edman degradation for N-group analysis.

Major Findings: When bradykinin was given rapidly I.V. to a rat, tachypnea and a diuresis occurred and 18.1% of the injected radioactivity was recovered from the urine in the first 5 hours, 19.3% in the first 24 hours. If the bradykinin was given slowly so there were no apparent physiologic effects 16% of the injected radioactivity was recovered in the first 6 hours, 15.8% in expired air and only 0.2% in the urine. When the bradykinin was given to a mouse I.P. and expired air and excreta were counted as well as the supernatant of a homogenate of the whole animal 69% of the injected radioactivity was recovered in the first 24 hours, 44% in expired air, less than 1% in excreta, and 25% in the animal homogenate. The rate of disappearance of total radioactivity was determined by homogenizing whole mice at various times after I.P. injection of bradykinin and then measuring total radioactivity by a combustion technique on the whole homogenate. Using this method 70% of the radioactivity had a half-life of 14 hours, and 30% had a half-life of 60 hours or more.

In 2 patients given bradykinin I.V. slowly to avoid any apparent physiologic effects, 13.4 and 9.4% of the injected radioactivity was recovered in the first 24 hours, most in the first hour. Seventy-five to 85% of this appeared as a single peak on column chromatography. Acid hydrolysis and enzymatic hydrolysis of this peak showed that all the counts were present in proline and an Edman degradation indicated that the N-terminal group was proline. There was not sufficient material to do a direct amino-acid analysis of the peptide present in this peak.

Significance: These data confirm in a new way that bradykinin is rapidly metabolized. They indicate that with rapid infusion a significant amount of a metabolite is present in the urine. When infusion is slower and more physiologic there is little metabolite in the urine but a great deal is completely degraded and appears as CO_2 in the expired air. Significant amounts of bradykinin must be broken down to the original amino acids with proline then entering the general protein pool. This would explain the long half-life of 1/3 of the material.

The data from human studies indicate that the radioactive metabolite is a small peptide with proline in the N-terminal position.

Proposed Course of Project: 1. We are presently chromatographing authentic peptides of the possible peptide sequences from bradykinin to determine the identity of the major excretion product we have isolated.

2. We will perform more human studies to look for uniformity of excretion pattern.

3. When equipment is available, we will collect expired air in patients given radioactive bradykinin to better evaluate total recovery and total biologic half-life.

4. We will study variations in bradykinin metabolism in patients with various diseases.

Honors and Awards: None

Publications: None

Serial No. -NHI-249
1. Experimental Therapeutics Branch
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Antihypertensive Properties of 3,5-dihydroxy-4-methoxyphenylalanine.

Previous Serial Number: None

Principal Investigator: Sydney Spector, Ph.D.

Other Investigators: Ryo Tabei, M.D., John Daly, Ph.D., Cyrus Creveling, Ph.D. Bernard Witkop, Ph.D. and Albert Sjoerdsma, M.D., Ph.D.

Cooperating Units: Laboratory of Chemistry, NIAMD

Man Years

Total: .10
Professional: .10
Other: 0

Project Description:

Objectives: Many amino acid derivatives, such as α-methyl-dopa and α-methyl-m-tyrosine, can deplete endogenous tissue norepinephrine. It had been reported that 4-methoxy-3,5-dihydroxyphenethylamine depletes cardiac norepinephrine and since the amine could be formed by the decarboxylation of 3,5-dihydroxy-4-methoxy-phenylalanine, studies were undertaken with the amino acid to answer the questions whether the amino acid derivative depletes tissue norepinephrine and whether it can lower blood pressure.

Methods: Tissue content of norepinephrine was assayed either by the extraction procedure of Mead and Finger or by the trihydroxyindole method. Blood pressure effects were evaluated using the spontaneously hypertensive rats which had been selectively produced by Okamoto and Aoki and now being bred at the NIH.

Major Findings: A rapid reduction of endogenous norepinephrine occurs in brain and heart following a single intraperitoneal injection of 35 mg/kg of 3,5-dihydroxy-4-methoxy-phenylalanine and returns to control values after 72 hours. Although the compound failed to lower the blood pressure of the normotensive rat, when administered to the spontaneously hypertensive rats there occurred a reduction in the blood pressure of about 20 mm Hg. The effects of a single dose (35 mg/kg i.p.) persisted for 24 hours. The effects of the compound are reversible, for with discontinuance of the drug for 48 hours the blood pressure

154

reverted back to control hypertensive levels.

Significance & Proposed Course of Project: This new amino acid exerts an anti-hypertensive effect on the spontaneously hypertensive rat which is more marked than that obtained with α-methyl-dopa. Also, the compound failed to exhibit any apparent toxicity, suggesting potential use in man.

Honors and Awards: None

Publications:

1. Spector, S., Tabei, R., Daly, J., Creveling, C., Witkop, B. and Sjoerdsma, A.: Reduction of tissue norepinephrine and blood pressure by 3,5-dihydroxy-4-methoxy-phenylalanine. Life Sciences. In press.

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART INSTITUTE
July 1, 1967 through June 30, 1968

The Laboratory of Kidney & Electrolyte Metabolism is currently engaged in the following groups of studies: 1. *In vitro* examination of water and solute transport across isolated segments of renal tubule, 2. *In vivo* analysis of renal function in the intact animal utilizing micropuncture technique, 3. Water and electrolyte transport and the intermediary metabolism of the toad bladder, particularly insofar as they relate to the action of certain hormones, 4. Transport of electrolytes in the red cell and characterization of membrane components, 5. Immunochemical factors in renal disease, and 6. Tension-frequency studies in cardiac and skeletal muscle.

I. The isolated perfused renal tubule in the rabbit.

The technique for isolation and *in vitro* perfusion of individual segments of the rabbit renal tubule has been described in earlier reports. It has been demonstrated that vasopressin, its intracellular intermediate cyclic-AMP, and theophylline each increase osmotic flow of water across the collecting tubule. The effect is associated with characteristic anatomical changes; namely, swelling of the epithelial cells and dilatation of lateral intercellular spaces. On electron microscopy, the dilated lateral spaces are open at the basal surface of the cell and tightly sealed at the apical border.

Efforts this year have been directed at determining the pathway for bulk flow of water from tubule lumen to blood. Vasopressin increases the permeability of the luminal membrane to water which then can flow into the cell as evidenced by swelling of the tissue. The water undoubtedly leaves by direct passage across the basal surface but in addition crosses the lateral border, enters the interspace and ultimately exits into the bathing medium through the visible opening at the basal end of the dilated channel. Unlike other tissues (e.g. gall bladder) the osmotic force promoting flow into the interspace is not developed by active transport of Na^+ since ouabain, a potent inhibitor of sodium transport neither abolishes the hydro-osmotic effect of vasopressin nor prevents dilatation of the interspace. It is likely that the space is in functional communication with the extracellular compartment and that diffusion of solute from

69b

the latter into the space provides the necessary osmotic gradient. Evidence that the lateral spaces are direct extensions of the extracellular compartment was obtained in studies utilizing horse radish peroxidase. The enzyme was observed in the interspace of the collecting tubule only when added to the basal bathing medium.

In toad bladder and frog skin, urea and certain analogous compounds, i.e. thiourea and acetamide, are thought to traverse the same aqueous channels in passage across the tissue as does water. This conclusion is based on the so-called solvent drag phenomenon in which the unidirectional flux of the pertinent solute is accelerated in the direction of osmotic water flow and decelerated in the reverse direction. In earlier studies the diffusional permeability of the collecting tubules to urea was found to be extremely low, was unaffected by antidiuretic hormone, though the hormone did increase the diffusional permeability to water. In the past year these studies have been extended to include an examination of the unidirectional flux of urea, thiourea and acetamide during net flow of water induced by vasopressin. The permeability of the collecting tubule to each of these compounds was extremely low in the absence of water flow and was unaltered by vasopressin-induced osmotic flow. On the basis of these results it is likely that the specific solutes investigated permeate the collecting tubule by a path distinct from that traversed by water.

The electrical characteristics of the isolated perfused collecting tubule have been studied in considerable detail. A resting potential difference across the membrane is present which is reduced by vasopressin and ouabain. This contrasts with findings in the isolated proximal tubule of the rabbit in which no potential difference is evident despite reabsorption of water and electrolyte. Membrane resistance of the collecting tubule, calculated from the length constant determined by passage of known quantities of current into the tubule, was found to be high and consistent with the known low ionic permeabilities of this portion of the nephron. This also contrasts with the proximal nephron in which a shorter length constant and lower resistance have been reported by others. In the past year refinements in technique have permitted more detailed analysis of the time course and magnitude of the changes in electrical phenomena. Specifically, it is now possible to insulate both ends of the perfused tubule with a resin and thereby to obtain more accurate measurements of P.D., resistance and length constant. On the basis of the most recent studies it has been found that

application of constant strength current causes a progressive change in the potential difference over approximately 50 seconds without a concomitant change in membrane resistance. The significance of this so-called "creep" phenomenon, its relationship to ionic distribution, etc., is being studied.

As indicated above the isolated perfused proximal tubule of the rabbit does not manifest a potential difference despite otherwise "normal" salt and water reabsorption. Furthermore net reabsorption is unaffected by the addition of the hormone angiotensin or by changing the radius of the tubule (see below). Bidirectional sodium fluxes, osmotic permeability coefficients of the membrane, and the effective osmotic pressure of sodium chloride have now been determined. It is apparent that the tubule wall in this segment as contrasted with the collecting tubule is relatively leaky to salt and that the reflection coefficient for sodium chloride (the ratio of the observed osmotic pressure to that measured cryoscopically) is approximately 0.7. These results are consistent with *in vivo* results of others. Studies of the effects of diuretic agents on the reabsorptive capacity of the tubule are in progress.

Transport of the weak electrolytes PAH, diodrast and chlorphenol red have been studied in both isolated flounder and rabbit proximal tubule. These comparative studies have permitted a number of conclusions concerning the mechanism of transport of weak acids in the two species. In the rabbit, active secretion of PAH from bath to lumen is evident in both the convoluted and straight segments of the proximal tubule. Interestingly, the rate of transport in the straight segment is considerably greater than in the convoluted portion. In both segments the concentration of PAH in cell water exceeds that of bathing medium and lumen fluid. These and other observations have led to the conclusion that PAH is transported actively into the cell from the bathing medium and then diffuses passively from the cell into lumen fluid. There is little effect of changes in perfusion rate on the rate of transport of PAH indicating (1) that the rate limiting step in the process of secretion is active transport into the cell, and (2) that diffusion of the weak acid from lumen to bathing medium is minimal. The latter has been confirmed by direct experimentation.

In contrast to the rabbit, studies in the flounder have revealed that though PAH, diodrast and chlorphenol red are actively secreted into the lumen the concentration profile differs

for the three compounds. In the case of chlorphenol red, apparent cell concentration is less than in tubule fluid, whereas the cell concentrations of diodrast and PAH are respectively higher than and equal to that in the bathing medium. On the basis of these preliminary observations it is likely that two active transport steps, one across the basilar border, the other across the luminal border, may be involved in the transport of chlorphenol red. The characteristics of glucose and amino acid absorption are also being examined.

II. Micropuncture Studies.

Micropuncture studies in rat and dog in this laboratory are directed in large part at determining those factors responsible for the regulation of sodium excretion in the mammal. A number of manipulations interfere with sodium reabsorption in the proximal nephron; infusion of saline, expansion of blood volume either with blood or hyperoncotic albumin, dilution of the hematocrit, etc. With the exception of saline infusion none of the other manipulations results in major increases in sodium excretion. Expansion of blood volume per se in the dog significantly reduces proximal sodium reabsorption as estimated by a fall in TF/P inulin ratio. Similar changes accompany dilution of the hematocrit and infusion of saline. Only in the case of expansion by the first of the three manipulations are there associated changes in renal blood flow and filtration fraction. The changes noted, a decrease in blood flow and a rise in filtration fraction, are postulated by others as responsible for enhanced proximal reabsorption rather than the decrease observed in these studies.

Extracellular fluid contraction has also been studied. Contraction produced by peritoneal administration of polyethylene glycol results in enhanced proximal reabsorption in the rat. In the same studies in addition to estimating reabsorption from the TF/P inulin ratios during free flow of urine, the Gertz shrinking drop technique was employed. This technique depends on measuring the rate of reabsorption of a drop of saline placed between two samples of oil in a blocked tubule. The results were consistent with those obtained using the free-flow inulin ratios.

The Gertz technique has also been applied to an investigation of the so-called "third" factor. This is thought to be a humoral substance responsible for the development of the marked diuresis which follows expansion of hydropenic animals with saline. It

has been reported that plasma obtained from diuresing animals inhibits proximal reabsorption in a recipient rat when estimated by the Gertz technique whereas control plasma from a hydropenic donor is without effect. Attempts to confirm this important observation in this laboratory have thus far been unsuccessful.

At least two hypotheses have been proposed to explain the adjustment in sodium reabsorption which follows changes in filtration rate ("glomerulotubular balance"). In general it has been assumed that fractional reabsorption of fluid in the proximal nephron is maintained relatively constant despite changes in filtration rate. This relative constancy has been ascribed (1) to an intra-renal humoral feedback mechanism and/or (2) to an effect of tubular volume (radius) on the absorptive rate. Both hypotheses, the feedback thesis and the regulation of reabsorption by changes in the geometry of the tubule have been tested in the intact rat and in preliminary studies in the dog. In contrast to earlier inferences, it has been observed in this laboratory that absolute constancy of reabsorption as determined by the inulin TF/P ratio is not obtained in the rat when filtration rate is altered by constriction of the aorta and its subsequent release. Fractional reabsorption in the rat rises following constriction and falls following release. Despite this, some degree of balance is maintained since in most instances absolute reabsorptive rate changes in parallel with filtration rate. The changes in TF/P inulin were observed within 1 minute of the alteration in blood pressure induced by constriction or release of the clamp and were essentially unchanged 5 minutes later. The rapidity of the adjustment and its relative constancy are interpreted as contrary to the humoral feedback thesis.

In the same experiments the effects of tubule geometry on reabsorption were estimated. It has been stated that dilatation of the tubule (i.e. an increase in radius) results in an augmented rate of proximal reabsorption whereas the reverse occurs when tubule radius diminishes. This was evaluated by measuring the TF/P inulin ratio, the transit time for dye passage through the tubule, and volume flow of urine in the proximal nephron. From these data, the reabsorptive rate and tubule radius can be calculated. It was observed that changes in reabsorption did not parallel the calculated changes in tubule radius, nor was any degree of proportionality noted. Similar conclusions were derived from studies in which the same measurements were made prior to and during periods of increased ureteral pressure. It

690

has been concluded that reabsorption rate is not governed by tubule geometry. Similar conclusions were reached in studies employing the isolated perfused proximal tubule of the rabbit (see above).

In last year's report preliminary results of studies of in vitro perfusion of the rat papilla were described. These studies have been extended. Although it has not been possible as yet to demonstrate active sodium transport in any portion of the loop of Henle examined, certain important conclusions were derived from these studies. The diffusional and osmotic permeability coefficients of water and the diffusional coefficient of urea were measured in all segments of the loop of Henle and the collecting duct. The descending limb of the loop was found to be two to three times as permeable to water as the ascending limb and the collecting duct, whereas the permeability to urea was approximately equal in all segments. Net water movement along an osmotic gradient in the absence of vasopressin was appreciable only in the permeable descending limb. Antidiuretic hormone increased both the diffusional and osmotic permeability to water of the collecting duct and had no effect in the loop. The permeability to urea of the collecting duct was also significantly increased by vasopressin. The osmotic effectiveness of urea, sodium chloride and mannitol was also determined. In all structures urea was less effective than equiosmolal mannitol and readily entered the lumen. On the other hand sodium chloride and mannitol were equally effective osmotically in all segments with the exception of the descending limb. In the latter, sodium entered the lumen whereas mannitol did not and its osmotic effectiveness was approximately 30% of that of mannitol. The results are consistent with current views concerning the role of the loop of Henle and the collecting ducts in the mechanism of urine concentration and dilution.

In an effort to study regulation of sodium reabsorption by the loop of Henle, in vivo microperfusion studies have been initiated in the rat. Fluid injected into the proximal nephron is collected in the early distal segment and analyzed. Although the experiments are still in their initial stages, it appears that the systemic administration of saline does not alter sodium reabsorption in this portion of the nephron whereas it decreases reabsorption in the proximal segment. The diuretic agent furosemide on the other hand inhibits reabsorption of sodium in the loop of Henle.

III. Studies in the Toad Bladder.

The toad bladder responds to vasopressin, cyclic-AMP and theophylline with an increase in osmotic water flow and sodium transport. The permeability of the bladder to other substances such as urea is also increased by the hormone. It is currently accepted that antidiuretic hormone stimulates the enzyme adenyl cyclase which converts ATP to cyclic-AMP and that the latter derivative is responsible for the permeability changes. In view of this it is likely that intrinsic alterations in the activity of the enzyme *in vivo* may modulate the activity of the effector hormone (vasopressin) in this tissue and in the kidney. Observations relating to this thesis have been discussed in earlier reports. In the past year the effect of a variety of catecholamines on the hydro-osmotic response to vasopressin, cyclic-AMP and theophylline has been examined with this in mind. Both epinephrine and norepinephrine inhibit the hydro-osmotic effects of ADH and theophylline but not that of cyclic-AMP. This is interpreted as indicating that catecholamines decrease the activity of adenyl cyclase in toad bladder and thereby minimize activation of the enzyme by vasopressin. Inhibition of the theophylline response is presumably a consequence of a decrease in the steady state concentration of cyclic-AMP induced by epinephrine. The inhibition of adenyl cyclase activity by epinephrine is an α-adrenergic effect since it is blocked by the simultaneous administration of alpha blocking agents and not by beta blocking agents. Although under some circumstances beta adrenergic agents have been shown to potentiate the effects of vasopressin, a result compatible with the view that beta agents stimulate adenyl cyclase activity, other interpretations have not been excluded.

As indicated above cyclic-AMP, as does vasopressin, increases active sodium transport across the toad bladder. It is generally assumed that baseline active transport in non-stimulated tissue and the additional transport induced by cyclic-AMP reflect the activity of a single transport path. It is conceivable, however, that two separate paths exist, only one of which is activated by antidiuretic hormone although both may converge on a final common step. Evidence consistent with the latter view was obtained in experiments in which inhibitors of active sodium transport were employed. These included ouabain, chlorpropamide, fluoroacetate, ethacrynic acid, etc. Concentrations were selected to reduce baseline Na^+ transport approximately 50%. Subsequent addition of cyclic-AMP to inhibited tissue in all instances with the exception of ouabain-treated tissue, resulted in stimulation of

692

sodium transport indistinguishable from that observed in non-inhibited tissue.

Chlorpropamide, a sulfonylurea derivative, has been reported to be effective in the therapy of patients with hypothalamic and pituitary diabetes insipidus but not in patients with nephrogenic diabetes insipidus. It has been proposed that the drug interacts with vasopressin receptors in the kidney and thereby mimics vasopressin. This appears unlikely since the drug is ineffective in normal man undergoing water diuresis. Furthermore it has no effect on osmotic water flow across the toad bladder. Of interest however is the observation that chlorpropamide does potentiate the effect of both vasopressin and theophylline whereas it interferes with that of exogenous cyclic-AMP in toad bladder.

The effects of hyperosmolality of either the mucosal or serosal bathing medium of toad bladder on net water flux, diffusional permeability to water and the electrical characteristics of the tissue were examined using a variety of compounds. These included urea, certain of its analogs, sugars, sugar alcohols and a variety of inorganic salts. Considerable differences in the response of the tissue to mucosal hyperosmolality as contrasted with serosal hyperosmolality were observed. In general mucosal hyperosmolality induced by urea and some of its analogs markedly increased osmotic water flow and diffusional permeability to water in the absence of vasopressin as did sodium chloride. This was not noted with the other compounds tested, nor was there any correlation between the permeance of the compound selected and its hydro-osmotic effect. Changes induced by serosal hyperosmolality on the other hand were in general related to the permeance of the compound selected.

Aldosterone and other adrenal steroids have unique effects on the toad bladder. Evidence has been presented by a number of laboratories that the increase in sodium transport effected by these agents is a consequence of induction of protein synthesis secondary to stimulation of the formation of messenger RNA. Studies in this laboratory have been directed at examining the effect of the adrenal steroids on Na^+ transport and the hydro-osmotic response to vasopressin. It has been noted that net transport of Na^+ and the hydro-osmotic response of the toad bladder to ADH decline after overnight incubation. This is assumed to be a consequence of depletion of endogenous steroid since it can be prevented by pre-incubation with aldosterone. Similar effects on urea permeability have also been noted. In

order to separate the two phenomena and to determine whether they are mediated by different systems, studies were performed in which the differential effect of mineralocorticoids and glucocorticoids on water and salt transport were compared. On the basis of the studies it appears likely that the steroid-induced pathway mediating the enhanced water permeability response to vasopressin after overnight incubation differs from that mediating the effect on sodium transport. Thus it has been shown that glucocorticoids in concentrations which induce equivalent changes in Na^+ transport as certain mineralocorticoids are capable of eliciting a greater effect on the hydro-osmotic response to vasopressin. The reverse has also been shown. Of considerable significance is the observation that though spironolactone, an antagonist of the mineralocorticoids, depresses the sodium transport response to dexamethasone (a glucocorticoid) it does not alter its effect on the water permeability response to ADH. This appears to be the first clearcut demonstration of a primary effect of these agents on water transport across an epithelial structure.

In association with these experiments metabolic studies involving analysis of the concentration of substrates and products in the glycolytic chain have been performed. Aldosterone markedly stimulates glycolysis in toad bladder. The effect is dependent on active sodium transport in that no stimulation of glycolysis is observed following addition of ouabain. Aldosterone apparently alters glycolysis by affecting two rate controlling enzymatic steps, those catalyzed by phosphofructokinase and pyruvate kinase.

Although it has been suggested by others that aldosterone stimulates the production of ATP in toad bladder and thereby provides the substrate for the energy consuming process of active Na^+ transport no evidence favoring this view has been obtained in this laboratory. No change in the concentration of ATP in control and aldosterone treated tissues has been observed. However a significant decrease in the concentration of creatine phosphate and a rise in that of creatine were uniformly observed in bladders treated with the mineralocorticoid. It is likely that aldosterone stimulates sodium transport directly by an unknown mechanism and that the fall in creatine phosphate reflects increased utilization of high energy phosphate from ATP in the transport process.

It has been known that vasopressin increases the oxidation

of glucose in association with enhanced sodium transport. Information regarding fatty acid oxidation is sparse. In preliminary studies designed to examine the lipid composition of the tissue following hormone addition, it has been observed that enhanced sodium transport induced by vasopressin is reflected in an increase in the oxidation of labelled palmitate. The enhancement in palmitate oxidation is depressed by removal of sodium or by the addition of the inhibitor ouabain.

IV. Red Cell Studies.

Human erythrocyte ghosts are hemoglobin-free cells which retain many of the transport properties of the intact cell. They afford a unique opportunity for studying the plasma membrane in isolation. In the past year it has been observed that if hemolysis is effected under specific conditions, the ghosts fragment into two major fractions: (1) a soluble protein in the supernatant and (2) a particulate portion made up of numerous vesicles. The soluble protein which may represent the structural protein of the intact cell membrane can be made to gel on addition of salt. Under these circumstances it has a characteristic appearance on the electron micrograph. With the aid of a negative staining technique a fibrous system is visualized which resembles that reported in intact ghost membranes. In addition the fibrous portion contains Na-K insensitive ATP-ase activity. The vesicles are surrounded by a clearly defined unit membrane. Furthermore they retain osmometric properties reminiscent of the parent structure, can be made to aggregate and in addition retain an important property of the intact membrane, i.e. Na-K activated ATP-ase activity. It is hoped that further characterization, particularly of the vesicular fraction of the disrupted red cell ghost, may provide important information regarding the molecular basis of electrolyte transport in the intact tissue.

In association with these studies, an investigation of the mechanism of volume regulation in avian erythrocytes has been initiated. To date it appears that cells whether swollen by incubation in demineralized water or shrunken with hypertonic KCl, rapidly regain their initial volume by appropriate alterations in electrolyte content. The changes are unaffected by ouabain.

The contractile vacuole of the giant amoeba is thought to be involved in osmoregulation. The vacuole is hypotonic to cytoplasm, progressively increases in size and then is expelled from the amoeba. It has been postulated that hypotonicity is

achieved by active extrusion of electrolyte in excess of water as cytoplasmic fluid continues to enter the growing vacuole. On the basis of studies performed in the past year this appears unlikely. It has been noted that vacuoles grow by coalescence but that their osmolality is constant and independent of size. It is likely that isosmotic pre-vacuoles (vesicles) become hypotonic by appropriate adjustments in electrolyte transport and that coalescence of these hypotonic units occurs subsequent to achievement of final osmolality.

V. Immunochemical Studies of Nephritis.

The onset of a class of glomerulonephritis in experimental animals is assumed to be associated with the appearance of circulating soluble antigen-antibody complexes. In human nephritis affected glomeruli may contain antigen-antibody complexes which by analogy are thought to be derived from the circulation. This has not been established nor has the presence of circulating complexes been demonstrated with certainty in the human disease.

Methods for the detection of minute quantities of these complexes in plasma are being developed in order to evaluate their role in the development of renal disease in man. It is assumed that the circulating complex consists of antigen, immune globulin, and the C'3 fraction of complement. Sephadex fractionation permits separation of the large complex from free IgG globulin and C'3 in plasma. A radioimmunoassay method for detection of the minute quantities of C'3 in the complex has been developed and efforts are now being directed at developing a sensitive technique for the assay of the antibody component. It is proposed to employ this system in appropriate disease states and control subjects in the coming year.

VI. Studies in Cardiac and Skeletal Muscle.

A detailed study of the factors controlling the contractility of heart and skeletal muscle is in progress. Myocardial muscle of all animals exhibits the Bowditch staircase phenomenon, i.e. the increase in contractility which accompanies increasing rates of stimulation. In muscle from most mammals maintenance of contractile force is achieved both by the Bowditch phenomenon and the Woodworth reverse staircase phenomenon. The latter is the increase in contractility which occurs at diminishing frequencies of stimulation. The two systems complement each other in the sustenance of contractile force over a wide range of stimulation

frequency. It has been observed in this laboratory that the alkaloid Ryanodine specifically abolishes the Woodworth phenomenon in myocardial tissue without affecting other characteristics of the muscle. In the past year the reverse-staircase effect has been demonstrated in skeletal muscle of a number of species including rat and frog. It has been shown that Ryanodine also abolishes the phenomenon in skeletal muscle and that elimination of contraction under these circumstances is associated with a loss of calcium^{45} from pre-labelled tissue. Further evidence of the importance of calcium in the contractile process was obtained when it was noted that abolition of the Woodworth phenomenon may be produced by stimulation of muscle over long periods in a medium free of calcium. Restitution is achieved rapidly by re-introduction of calcium into the medium. This and other observations have led to the conclusion that bound calcium is a necessary requirement for electromechanical coupling. It is assumed that in skeletal muscle as well as in mammalian cardiac muscle calcium is freed from its bound state in the interval between contraction and becomes available for the development of tension during electrical depolarization.

Serial No. NHI - 250
1. Kidney & Electrolyte Metabolism
2. Renal Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Electron microscope studies of isolated collecting tubules

Previous Serial Number: NHI - 243

Principal Investigators: Jared Grantham, M. D.
Maurice B. Burg, M. D.
Jack Orloff, M. D.

Other Investigators: Charles Ganote, M. D.

Cooperating Units: Laboratory of Experimental Pathology
National Institute of Arthritis and Metabolic Diseases

Man Years:
Total: 7/12
Professional: 6/12
Other: 1/12

Project Description:

Objectives: To determine the pathway of "osmotic" water flow across the walls of rabbit collecting tubules.

Methods: Techniques for routine fixation of isolated perfused renal tubules of rabbits have been described. In the present studies it was found that tubules swollen in dilute media were more permeable to glutaraldehyde than tubules incubated in normal strength Ringer's. In a series of experiments it was determined that further swelling during fixation could be prevented if the Na content of the dilute medium was replaced with glutaraldehyde (1%) in the approximate proportion of 127 mOsm glut. $\approx$ 20 mEq Na. We also observed that fixed tubules retained some capacity to swell and shrink in dilute and concentrated salt solutions, respectively. This necessitated careful regulation of osmolality of all postfixation and rinsing solutions. Alcohol dehydration caused fixed specimens to shrink approximately 30%; an alternate method of dehydration which does not shrink fixed tissue has not been found.

Horseradish peroxidase was used as a tracer in order to determine whether the slit at the base of the intercellular spaces admits substances of large molecular size.

Major Findings: In earlier studies ADH caused widening of the lateral intercellular spaces as well as swelling of the cells of tubules perfused with hypotonic solution. In the present studies swelling of the cells by immersion

698

in dilute bathing medium produced no widening of previously collapsed intercellular spaces. Treatment of perfused tubules with 5×10^{-5} M ouabain, a concentration of drug shown previously to decrease Na efflux and reduce transtubule PD to zero, did not interfer with the usual morphologic response to ADH. Transferring swollen tissue back to normal strength Ringer's resulted in immediate shrinkage of the tissue with translocation of some of the excess cell water into the lateral intercellular spaces. The widened channels returned to their normal collapsed state within 1-5 minutes. Increasing transtubular pressure by only 5 cm H_2O reduced the collapse time of the spaces to 10-20 seconds. Horseradish peroxidase was found in the intercellular channels when the enzyme was placed in the outer bathing medium only.

These findings are interpreted to indicate that ADH permits water to flow into the cytoplasm through the apical portion of the cell. Water moves from the cell into the bath across the lateral membranes and through the basilar slit as well as directly across the basal surface. The flow of water across the basal and lateral cell surfaces, as opposed to the lumen membrane, proceeds independently of ADH. The driving force for osmotic water flow across the lateral membranes is provided by the passive diffusion of Na from the bath into the interspace.

Proposed Course of Project: Quantitation of the relative volume flows across the basal and lateral cell surfaces. The role of the basement membrane in the transport of water will be evaluated.

Honors and Awards: None

Publications: Ganote, C. E., Grantham, J. J., Moses, H. L., Burg, M. B. and Orloff, J.: Ultrastructural studies of vasopressin effect on isolated perfused renal collecting tubules of the rabbit. J. Cell. Biol. 36: 355-367, 1968.

Serial No. NHI - 251
1. Kidney & Electrolyte Metabolism
2. Renal Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Function of isolated perfused rabbit proximal tubules

Previous Serial Number: NHI - 240

Principal Investigators: Juha P. Kokko, M. D.
Maurice B. Burg, M. D.
Jack Orloff, M. D.

Cooperating Units: None

Man Years:
Total: 19/12
Professional: 16/12
Other: 3/12

Project Description:

Objectives: Study mechanism of sodium and water transport in proximal renal tubule.

Methods: Methods for dissection and perfusing isolated rabbit tubules were previously described. For the present studies instead of the artificial physiological saline solutions formerly used, the tubules were dissected and bathed in rabbit serum and perfused with an ultrafiltrate prepared from the same serum. Fluid absorption was estimated from changes in the concentration of albumin I^{125} included in the perfusate.

Major Findings:

1. Techniques for bidirectional Na^+ flux studies have been developed. Preliminary results indicate that at a perfusion rate of 6 nl/min/mm of tubule length some 2/3 of original intraluminal sodium exchanges without change in concentration. Studies of the effect of diuretics on the sodium transport mechanism are under way.

2. Preliminary results indicate that mercurial diuretics do not affect water absorption by the proximal tubule of the rabbit, whereas furosomide is inhibitory.

3. The reflection coefficient for Na was measured by perfusing proximal tubules with raffinose solution iso-osmolal to the bath of rabbit serum (300 mOsm). Na^{22} was placed in the external bath for measurement of net influx of

700

sodium into tubule lumen. The final intraluminal sodium concentration was an inverse function of the perfusion rate. The osmolality of the collected tubule fluid was always slightly greater than that of the perfusion solution and the bathing media, and there was net water movement into the lumen. When the bath was made hypertonic to the intraluminal raffinose solution by addition of NaCl, it was found that net water movement was reduced to zero at a bathing medium osmolality of 405 mOsm. This indicates that the reflection coefficient for NaCl is 0.68 in good agreement with the previous result of .67 for this tissue by a different method.

Proposed Course of Project: In these initial studies it has been established that the isolated proximal renal tubule transports salt and water actively in vitro despite a high sodium permeability and low sodium reflection coefficient. This is in agreement with previous studied in vivo, and suggests that the normal Na transport mechanism remains operative in vitro. With this new preparation it is possible to control experimental conditions more precisely than in vivo as well as to measure cellular Na and water contents. With this method it is proposed to investigate the nature of this important transport mechanism. Further, it should be possible to study the mechanism of action of clinically important diuretics.

Honors and Awards: None

Publications: None

Serial No. NHI - 252
1. Kidney & Electrolyte Metabolism
2. Renal Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of electrolyte transport in isolated perfused collecting tubules

Previous Serial Number: NHI - 241

Principal Investigators: Jared J. Grantham, M. D.
Maurice B. Burg, M. D.
Jack Orloff, M. D.

Other Investigators: Clifford Patlak, Ph. D.

Cooperating Units: Biometry Branch
National Institute of Mental Health

Man Years:
Total: 2/12
Professional: 2/12
Other: 0

Project Description:

Methods: Isolated collecting tubules of rabbits were perfused and bathed with solutions of different Na and K concentrations. Transtubule electrical potential (PD) at the perfusion end and Na and K concentrations of the collected perfusate were measured.

Major Findings: We attempted to test the earlier tentative conclusion that K transport was largely passive in character by changing the concentrations of K (substituting for Na) in both the bath and lumen solutions. When the concentration of K in the bath was raised from 5 mEq/L to 10 mEq/L (shown previously to cause no significant change in PD) the concentration of K in the collected fluid increased only 20%. This was clearly less than the 100% increase in concentration expected if K was passively distributed in accordance with the PD. In the earlier studies a perfusion rate of < 1 nl/min/mm tubule length (equal to or lower than the anticipated rate in this tubule segment in vivo) had appeared adequate to achieve maximal concentrations of K in the collected fluid, however in view of the newer results we reassessed the conditions necessary to attain K concentrations in the collected fluid which were independent of perfusion rate, i.e., the flow rate below which the net flux of K at the end of the tubule was reduced to zero. We attempted to decrease flow rate in stepwise fashion in order to determine the maximal concentration of K; this proved to be impossible owing to consistent increases of PD when flow rate (and tubule diameter) was decreased. In order to avoid

702

changing flow rate the lowest possible pump rate (Sage pump $\approx$ .2 nl/min) was used to perfuse tubules with concentrations of K higher than the anticipated equilibrium concentration. Results of 5 of these studies were scattered; however, in all experiments the collected K concentration was higher than that predicted from the observed PD (predicted 15.3, observed 35.0 mEq/L). These results were consistent with active secretion of potassium. At this point it was discovered in companion experiments designed to evaluate the electrical properties of collecting tubules that electrical insulation of the collecting end of the tubules was probably not adequate. Considerable effort was expended developing a practical method of insulating the ends of the tubule thus assuring constancy of PD along the entire length of tubule.

Proposed Course of Project: Using improved methods to perfuse and electrically insulate tubules the previous studies will be repeated at extremely low perfusion rates. Once the nature of the relationship between transport and potential is clarified we will proceed to evaluate the effects of various agents such as hormones and diuretics on electrolyte transport.

Honors and Awards: None

Publications: None

Serial No. NHI - 253
1. Kidney & Electrolyte Metabolism
2. Renal Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of water, urea and electrolyte transport in isolated perfused collecting tubules.

Previous Serial Number: NHI - 241

Principal Investigators: Sandy I. Helman, Ph.D.
Maurice B. Burg, M.D.
Jared J. Grantham, M.D.
Jack Orloff, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 11/12
Professional: 9/12
Other: 2/12

Project Description:

Objectives: To delineate the mechanism of water and solute permeation of renal tubules.

Methods: Isolated perfused collecting tubules were studied using methods previously detailed. Radioactive urea, acetamide and thiourea were perfused through the tubule lumen. From the amount of radioactive tracer appearing in the bath permeability was calculated. In some experiments, Sylgard 184 resin (Dow Chemical Company) was used to additionally seal both perfusion and collecting ends of the tubule against possible leaks.

Major Findings: Without the Sylgard resin sealing of the tubules in the pipets was slow and sometimes incomplete complicating the interpretation of the results. These problems were corrected by using the resin (which adhers strongly to both

glass and tissue to glue the cut ends of the tubule into the holding pipets. Mean urea, thiourea and acetamide permeability after a 150 to 180 minute incubation period were .92, 2.44 and 3.40 cm/sec x 10^{-6}, respectively. Addition of ADH to the bath resulted in a large increase in net H_2O absorption when an osmotic gradient was present. Mean solute permeabilities did not change with vasopressin indicating either that solute and water take separate paths through the membrane or that the pores that the water traverses are much smaller than in other membranes. Thermodynamic analysis indicates a reflection coefficient for these solutes used greater than 0.99.

Proposed Course of Project: Project is completed.

Honors and Awards: None

Publications: None

Serial No. NHI - 254
1. Kidney & Electrolyte Metabolism
2. Renal Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Electrical characteristics of collecting tubules

Previous Serial Number: NHI - 242

Principal Investigators: Sandy I. Helman, Ph. D.
Maurice B. Burg, M. D.
Jared J. Grantham, M. D.
Jack Orloff, M. D.

Cooperating Units: None

Man Years:
Total: 11/12
Professional: 10/12
Other: 1/12

Project Description:

Objectives: To measure electrical characteristics and ion transport properties of kidney tubules in order to characterize the mechanisms of electrolyte transport.

Methods:

1. Electrical system: A single perfusion pipet was used to deliver current and record potential on an oscilloscope. In order to measure resistance and to voltage clamp tubules it is necessary to electrically seal the cut ends of the tubule. Several methods were attempted to accomplish this. Electrical sealing of the perfusion end was accomplished by 1) using a perfusion pipet large enough to plug the lumen for 500-800 μ, 2) using a triple barrel concentric pipet arrangement such that the tubule cut end was pulled into mineral oil 3) similar to (2) except Sylgard 184 resin was used instead of mineral oil. Electrical sealing of the collecting end of the tubule was accomplished by 1) using a double barrel concentric pipet system and pulling the cut end into oil placed between the pipets or 2) more simply, a single pipet was used with Sylgard 184 Resin sealing the tubule in the tip of the pipet.

2. Calculations: Computer programs were written for analysis of the Electrical Core conductor equation such that from the measurements of applied current and voltage at the two ends of the tubule, values of length constant (λ), membrane resistance (rm) and core resistance (Rc) could be calculated.

Major Findings:

1. In preliminary experiments values of λ and Rm were in the range of 0.5 mm and 200 to 500 ohm cm^2 respectively. Calculated Rc varies with tubule diameter and agreed with the theoretical calculation of Rc (specific resistance of perfusion solution = 70 ohm cm^2).

2. Collecting tubules exhibited a "creep" phenomenon, i.e., with applied current, both depolarization and hyperpolarization of the tubule occurred for up to 50 seconds (depending on applied current strength, 10^{-8} to 8×10^{-8} amp) without an increase in membrane resistance.

Proposed Course of Project: 1) to delineate the mechanism of the creep phenomenon 2) to correlate tubule resistance and electrolyte permeability measured with radioisotopes 3) to determine the effect of voltage clamping and short circuiting on ion transport.

Honors and Awards: None

Publications: None

Serial No. NHI - 255
1. Kidney & Electrolyte Metabolism
2. Renal Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Transport of para-aminohippurate by the rabbit proximal tubule

Previous Serial Number: None

Principal Investigators: Bruce Tune, M. D.
Maurice B. Burg, M. D.
Jack Orloff, M. D.

Cooperating Units: None

Man Years:
Total: 19/12
Professional: 16/12
Other: 3/12

Project Description:

Objectives: To characterize the mechanism of transport of organic acids by the rabbit proximal tubule using para-aminohippurate ahd chlorphenol red.

Methods: Methods for dissecting and perfusing isolated rabbit tubules have been previously outlined. Tritiated PAH was placed in the bathing medium and net secretion into the tubule lumen was measured. Tissue concentration was determined by extraction of PAH in 3% trichloroacetic acid and weighing the subsequently dried tubules on a quartz fiber ultramicrobalance. Both convoluted and straight portions of the proximal tubule were studied during perfusion and in the non-perfused state. The straight portion of the proximal tubule was further studied by placing the tritiated PAH in the perfusing fluid instead of the bath to determine whether the kinetics of efflux conform to the model of transport suggested by the initial studies.

Major Findings:

Net Transport: Active secretion of PAH from bath to tubule lumen was found in both segments of the proximal tubule but was significantly greater in the straight than in the convoluted portion. In neither portion was the net transport significantly affected by changes in perfusion rate from 3 to 15 nl/min.

Tissue Levels: Tissue levels were consistently greater than tubular fluid levels of PAH, and both were generally greater in the straight than the convoluted portion. The tissue levels in the non-perfused isolated tubules were

758

also consistently greater in the straight portion than in the convoluted.

Gradient: Tissue and tubular fluid PAH concentrations fell similarly in both portions of the tubule with increasing rates of perfusion, and the coefficient of permeability of the luminal membrane appeared equal in both portions and constant within the range of perfusion rates studied.

Model: On the basis of the above findings a model of active uphill transport of PAH through the basilar membrane into the cells with subsequent passive diffusion down a concentration gradient into the tubule lumen was proposed. It was predicted that efflux of PAH originating in the tubular lumen would be insignificant in comparison with net secretion from bath to lumen. Also, it was predicted that tissue levels when the PAH originated in the lumen would approach but remain less than tubular fluid levels of PAH. Passive permeability coefficients were calculated for basilar and luminal membranes on the basis of the model and the data from the secretion studies.

Efflux: In proximal straight tubules perfused with PAH the efflux was small as predicted (or few percent of the previously measured net flux). Tissue levels also were slightly less than tubular fluid levels, as predicted, confirming the adequacy of the proposed model.

Chlorphenol Red: On the basis of studies of competition it had been concluded that PAH and chlorphenol red are transported by the same mechanism. In preliminary studies the uptake of chlorphenol red from the bathing medium by proximal straight tubules was examined. In the absence of flow of tubule fluid the dye appeared to be more concentrated in the lumen than in the cells (the lumen was kept open by blocking one end of the tubule and applying a slight hydrostatic pressure at the other). Whether this observation represents an actual step-up of concentration of the dye from cell-to-lumen (indicating a transport mechanism different from PAH)or a visual artifact, perhaps as a result of lower intracellular pH or an altered state of the dye within the cell is not clear at this point.

Proposed Course of Project:

1. To study the actual concentration of chlorphenol red in tissue and tubular fluid during perfusion using ultra micro spectrophotometric techniques.

2. To study the mechanism of transport of sugars and amino acids in the proximal tubule using similar techniques.

Honors and Awards: None

Publications: None

Serial No. NHI -256
1. Kidney & Electrolyte Metabolism
2. Renal Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of organic acid, sugar, and amino acid transport in isolated perfused flounder tubules

Previous Serial Number: NHI - 245

Principal Investigator: Maurice B. Burg, M. D.

Other Investigators: Peter Weller

Cooperating Units: Mt. Desert Island Biological Laboratory, Salisbury Cove, Maine

Man Years:
Total: 4/12
Professional: 2/12
Others: 2/12

Project Description:

Objectives: To elucidate the mechanism of transport of organic acids, sugars, and amino acids in renal tubules.

Methods: The methods were previously described. Single proximal renal tubules of the flounder Pseudopleuronectes americanus were perfused in vitro. Steady state concentration of transported substrates were determined in the external bathing solution, tubule fluid, and renal cells.

Major Findings: In 10 experiments the addition of PAH - H^3 (2×10^{-5} M.) to the external bath, resulted in secretion of PAH into the lumen at a rate (63.8×10^{-14} M. min^{-1} mm^{-1} tubule length) which is approximately equal to that previously observed for the same concentrations of iodopyracet (56×10^{-14}). The concentration of PAH in the tubule fluid was 21.6 times as great as in the bath, and the concentration of PAH in the cells approximately the same (18.0 times that in the bath). Thus, the concentration profile of organic acids during transport by flounder tubules varies, depending on the compound studied. For chlorphenol red the concentration appears to be much lower in the cells than in the tubule fluid, for PAH it is approximately the same, and for iodopyracet it is higher in cells than in tubule fluid. These results are explained by differences in the handling of various organic acids by two active transport mechanisms, one at the luminal and the other at the peritubular cell membrane. However, it is also possible that a portion of intracellular organic acid is bound and that this fraction differs depending on the compound studied or,

alternatively, that the chlorphenol red levels in tissue are incorrectly estimated by visual observation.

When inulin-C^{14} was placed in the external bath very little appeared in the perfusion fluid (final concentration in the perfusion fluid in 3 experiments .04 times that in the bath) indicating low permeability to inulin. Also, when inulin-C^{14} was placed in the perfusion fluid there was little change in its concentration (ratio of .90 collected/perfused in 9 experiments) indicating little net fluid movement.

When PAH-H^3 (3×10^{-4} M.) was placed in the perfusion fluid, there was some loss from the tubule lumen (mean PAH/inulin ratio in the collected fluid was .79 in 7 experiments). The PAH permeability measured by this efflux from the lumen is small, however, compared to the rapid rate of secretion into the lumen. Concentrations in the tissue of inulin and PAH originating from the lumen were also measured. The PAH concentration in the tissue was .57 times that in the perfusion fluid, whereas inulin in the tissue was .04 times that in the perfusion fluid. Thus, both the luminal and peritubular membranes provide significant barriers to PAH loss from the lumen.

Glycine transport was studied in 8 experiments using glycine-H^3 and glycine-C^{14}. Equal concentrations (5×10^{-5} M.) of glycine were placed in the perfusion fluid and external bath with a different radioactive label in each solution. Glycine concentration (estimated as the sum of C^{14} and H^3 activities) was .31 times as high in the collected as in the perfused fluid, consistent with net glycine absorption from the lumen. Movement of glycine from bath to lumen was small (concentration ratio .11, tubule fluid/bath). Tissue glycine concentration was 25 times greater than in the bath (or original perfusion fluid) suggesting that active transport into the cells from the lumen is a step in amino acid absorption. However, a large fraction of the glycine in the tissue (76%) originated from the bath suggesting that there is also transport into the cells across the peritubular border.

Glucose transport was studied in a similar manner. With 1.4 to 5.5 mM glucose in both the bath and perfusion fluid, glucose concentration in the fluid collected from the tubule lumen was .50 times that perfused, indicating net absorption (8 experiments). Movement of glucose from bath to lumen was small (concentration ratio .11, tubule fluid/bath). In contrast to the findings with glycine, however, concentrations of radioactivity in the tissue were relatively low and differed according to the position of the radioactive label in the glucose perfused. When uniformly labelled glucose was initially present in the perfusion fluid, total concentration of radioactivity in the tissue was 4.9 times that in the surrounding fluids (3 experiments), but with C-1 or C-6 labelled glucose this ratio was only 1.8 (5 experiments). The difference is due to the retention of products of glucose metabolism in the tissue from the uniformly labelled glucose, as was confirmed by paper chromatography.

When an isotonic Na-free perfusion fluid ($MgSO_4$ plus $CaSO_4$) was used, there was no inhibition of either glycine or glucose transport. This finding, however, does not rule out the possibility that the transport processes are

Na-dependent since Na entered the tubule lumen from the bathing medium in large quantities during perfusion. This was indicated by measurements with Na^{22} and also by the occurrence of net fluid movement into the lumen under these conditions (inulin ratio .67 collected/perfused in 4 experiments). This high Na permeability is comparable to that found in mammalian proximal tubules.

Proposed Course of Project:

1. To further investigate the observed differences in handling of various organic acids presumed to be transported by the same system in the flounder tubules.

2. To determine the nature of the passive permeability steps in organic acid transport, looking for possible carrier mechanisms.

Honors and Awards: None

Publications: None

712

Serial No. NHI - 257
1. Kidney & Electrolyte Metabolism
2. Renal Mechanisms
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Factors controlling sodium transport by the loop of Henle.

Previous Serial Number: None

Principal Investigators: Trefor Morgan, M.D.
Robert W. Berliner, M.D.

Cooperating Units: None

Man Years:
Total: 6/12
Professional: 6/12
Other: 0

Project Description:

Objectives: To study the control of sodium reabsorption by the loop of Henle.

Methods: Rats were prepared for micropuncture in the usual manner. The capsule was then removed from the kidney. The last surface convolution of the proximal tubule was entered and perfused with a 0.1% lissamine green saline solution. The proximal part of the proximal tubule was blocked with an oil column and the glomerular filtrate allowed to escape through a hole in the nephron. The first part of the distal tubule to appear was punctured and complete, timed collections were made. After completion of the perfusion all tubules were injected with latex, dissected out of the tissue and the site of infusion and collection were recorded.

1. The same tubule was perfused at varying rates to see the effect of altering sodium delivery on sodium transport.

2. Collections were made during hydropenic conditions and

713

then the animal was expanded with 10 mls of normal saline and further collections obtained. The fractional and absolute reabsorption before and after saline loading were compared. In each animal a proximal tubule segment was perfused before and after saline to observe if there was depression of proximal tubule reabsorption.

3. Loops of Henle were perfused with a solution containing 1 or 10 mgs litre^{-1} of furosemide and the amount of sodium reabsorbed was calculated. Furosemide was given intravenously to the animal and similar studies were performed.

4. Proximal tubule perfusion. The effect of collapsing or distending the proximal tubule was studied.

Major Findings:

1. As the amount of sodium delivered to the loop is increased the total amount of sodium reabsorbed is also increased but there is a decrease in fractional reabsorption.

2. Sodium reabsorption by the loop was not depressed by saline loading but a concomitant depression in proximal tubule reabsorption could be detected by this perfusion technique.

3. Furosemide was able to act at either the luminal or peritubular surface.

4. Tubular geometry appeared to have no controlling effect on proximal tubule reabsorption.

Proposed Course of Project:

The importance and meaning of these studies in the control of sodium excretion by the animal is being evaluated. We intend to continue this project and investigate further the control of Na reabsorption by the loop of Henle. We also propose to perfuse distal tubules and study their transport ability.

Honors and Awards: None

Publications: None

Serial No. NHI -258
1. Kidney & Electrolyte Metabolism
2. Renal Mechanisms
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Permeability of the medullary segments of the nephron *in vitro*. Evidence for the countercurrent hypothesis

Previous Serial Number: NHI - 246

Principal Investigators: Trefor Morgan, M. D.
Robert W. Berliner, M. D.

Cooperating Units: None

Man Years:
Total: 8/12
Professional: 8/12
Other: 0

Project Description:

Objectives: To study the permeability of the medullary segments of the nephron.

Methods: Using an *in vitro* preparation of an isolated rat papilla (described in last year's report) the permeability properties of the loop of Henle, collecting ducts and vasa recta were studied. The descending limb, the expanded portion of the descending limb, the ascending limb of the loop of Henle, collecting ducts and vasa recta were perfused with a lissamine green containing solution and collections made at a distal site. The perfusion rate, the distance perfused and the diameter of the structure being perfused were measured and from the fall in concentration of an isotope and the change in perfused volume the unidirectional fluxes of various substances and hence the permeability coefficient in the absence of volume change could be calculated. The net movement of water was calculated from the change in inulin concentration. Using this technique the unidirectional flux was found for urea and THO and the net movement of water calculated.

Major Findings: Under iso-osmotic conditions there was no net movement of water but when there was an osmolality difference there was net movement of water.

	Permeability Coefficient cms sec^{-1} x 10^{-5}		Net Water Flux nl cm^{-2} $mosm^{-1}$ min^{-1}
	Water	Urea	
Descending Limb	112	16	58
Expanded Portion of Descending Limb	46		
Ascending Limb	46	15	4·4
Collecting Duct	45	20	4·2
Collecting Duct (ADH)	87	30	30
Vas Rectum	194	58	41

Antidiuretic hormone did not affect the permeability of the loop of Henle to water or urea.

In a different series of experiments the osmolality difference between the two solutions was due to mannitol, NaCl or urea. This allowed us to compare the relative abilities of these substances to remove water and also their relative rates of entry into the lumen. In the collecting duct and ascending limb sodium chloride removed as much water as mannitol and only a small amount of sodium chloride entered the lumen. In the descending limb sodium chloride readily entered the lumen and it removed only 1/3 as much water as did mannitol. In all structures urea removed less water than did mannitol and a considerable amount of urea entered the luminal fluid.

These permeability data are what would be expected if a countercurrent system driven by a sodium pump in the ascending limb of Henle's loop were present in the papilla of the rat. A search for this pump has been made in this tissue but has been unrewarding. However, we have also not been able to demonstrate a sodium pump in the collecting duct and suspect that some essential substrate or hormone is lacking from our media.

Proposed Course of Project: Further work on this project has been temporarily shelved but we intend to search for the sodium pump again in the future.

Honors and Awards: None

Publications: Morgan, T., Sakai, F., and Berliner, R. W.: In vitro permeability of medullary collecting ducts to water and urea. Am. J. Physiol. 214: 574-581, March 1968.

Morgan, T. and Berliner, R. W.: Permeability of the loop of Henle, vasa recta and collecting ducts to water, urea and sodium. Am. J. Physiol. In press.

Serial No. NHI - 259
1. Kidney & Electrolyte Metabolism
2. Renal Mechanisms
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of decreased sodium reabsorption by the proximal tubule after vascular volume expansion

Previous Serial Number: None

Principal Investigators: Fred S. Wright, M. D.
Stuart S. Howards, M. D.
Franklyn G. Knox, M. D.
Robert W. Berliner, M. D.

Cooperating Units: None

Man Years:
Total: 6/12
Professional: 6/12
Other: 0

Project Description:

Objectives: The study was planned to explore the renal mechanisms involved in the decrease in fractional sodium reabsorption by the proximal tubule known to occur after infusion of hyperoncotic albumin solution. Mechanisms possibly involved in such a change are increased nephron filtration rate and decreased absolute sodium reabsorption. Such a change in epithelial sodium transport might be due to changes in renal hemodynamics, or to the presence of a hormone inhibiting reabsorption. In assessing the relative contribution of these factors to changes in sodium reabsorption we have used methods not previously applied to micropuncture experiments in dogs including measurement of the fractional rate of reabsorption by the shrinking drop method, measurement of individual nephron GFR and proximal transit time and measurements of proximal tubule volume.

Methods: Mongrel dogs were anesthetized and prepared for micropuncture. Glomerular filtration rate, renal blood flow, hematocrit, arterial blood pressure, and plasma and urine sodium concentration were measured.

The inulin concentration and volume of free flow micropuncture samples were measured. During these collections the passage time of dye from glomerulus to the point of micropuncture was measured. Photographs of the green dye in tubules were used to measure tubule diameter. These sampled tubules were injected with latex and measured after microdissection. The rate of reabsorption of a drop of saline isolated between oil columns in the proximal

117

tubule was measured from series of photographs. The diameter of these tubules was taken from the diameter of the oil columns.

All of these measurements were made before and after the administration of hyperoncotic albumin solution. Values could then be calculated for nephron filtration rate, fractional sodium reabsorption (% of filtered sodium) absolute sodium reabsorption, and sodium excretion. In addition, the absolute reabsorptive rate/tubule volume ($C/\pi r^2$) could be calculated from data obtained either by free flow micropuncture or by shrinking drop measurements in the same kidney.

Major Findings: As previously reported infusion of hyperoncotic solutions results in decreased sodium reabsorption by the proximal tubule with only small increases in sodium excretion. This is associated with a large increase in plasma volume. Preliminary results show no increase in nephron GFR and a decrease in absolute sodium reabsorption whether calculated from free flow or shrinking drop data. Calculated dimensions of the proximal tubule indicate that previous visual measurements are underestimates probably because of an optical artifact.

Proposed Course of Project: Additional experiments.

Honors and Awards: None

Publications: None

718

Serial No. NHI - 260
1. Kidney & Electrolyte Metabolism
2. Renal Mechanisms
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of hyperoncotic infusions on proximal tubule sodium reabsorption and renal sodium excretion in the dog

Previous Serial Number: None

Principal Investigators: Stuart S. Howards, M. D.
Franklyn G. Knox, M. D.
Fred S. Wright, M. D.
Robert W. Berliner, M. D.

Cooperating Units: None

Man Years:
Total: 6/12
Professional: 6/12
Other: 0

Project Description:

Objectives: To evaluate the effect of hyperoncotic infusions on the reabsorption of sodium in the proximal tubule and sodium excretion in the urine in order to gain added insight into the mechanisms controlling renal sodium excretion in hydropenic and diuretic states.

Methods: Two types of experiments were performed using mongrel dogs. (I). Recollection micropuncture experiments in which samples from proximal tubules were obtained during hydropenia and again from the same tubules following infusion of a test solution. The infused solutions included hyperoncotic albumin, hyperoncotic dextran, isotonic saline and a control solution equal in volume and sodium content to the hyperoncotic infusions.

(II). Experiments in which a water diuresis was established and the change in free water clearance following infusion of hyperoncotic albumin or dextral solution was measured.

Proximal tubule sodium reabsorption was calculated from the tubular fluid to plasma inulin ratios. Blood volume changes were calculated from changes in hematocrit. Glomerular filtration rates, renal sodium excretion and urine flow rates were determined in all experiments. Free water and osmolar clearances were determined in water diuresis experiments. A radioisotopic method for determining inulin clearance in the presence of dextran was validated and used in those experiments in which the animals received dextran.

Major Findings: Infusion of hyperoncotic solutions resulted in depression of proximal tubule sodium reabsorption and little or no increase in sodium excretion. Infusions of isotonic saline which caused a similar decrease in proximal tubule sodium reabsorption effected an increase in sodium excretion which was significantly larger than that following the hyperoncotic infusions. Hyperoncotic infusions and saline infusions caused similar increases in distal delivery of sodium as calculated from the changes in glomerular filtration rate and proximal sodium reabsorption; however, only the saline infusion resulted in a large natriuresis thus suggesting the distal tubule determines final sodium excretion.

Proposed Course of Project: Project completed.

Honors and Awards: None

Publications: Howards, S. S., Knox, F. G., Wright, F. S., Davis, B. B. and Berliner, R. W.: Depression of fractional reabsorption by the proximal tubule of the dog without sodium diuresis. J. Clin. Invest. In press.

720

Serial No. NHI - 261
1. Kidney & Electrolyte Metabolism
2. Renal Mechanisms
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The relationship between glomerular filtration rate and sodium reabsorption by the proximal tubule of the rat nephron.

Previous Serial Number: NHI - 251.

Principal Investigators: B. M. Brenner, M. D.
C. M. Bennett, M. D.
R. W. Berliner, M. D.

Cooperating Units: None

Man Years:
Total: 24/12
Professional: 18/12
Technical: 6/12

Project Description:

Objectives: To test two of the hypotheses proposed to explain the adjustment in sodium reabsorption by the proximal tubule that follows a change in the rate of glomerular filtration (glomerulotubular balance).

Methods: Measurements were made of fractional sodium reabsorption by the proximal tubule under 2 sets of experimental conditions.

1. In order to evaluate the change in sodium reabsorption following abrupt changes in GFR measurements were made of inulin oncentration in tubule fluid and plasma prior to and following alterations in renal artery perfusion pressure produced by aortic constriction (clamp placed just above left renal artery) or release of constriction.

2. To determine the role of divergent changes in GFR and

tubule volume, measurements of proximal sodium reabsorption were made prior to and following elevation of ureteral pressure.

Major Findings: It has been suggested that glomerulotubular balance is mediated by a humoral feedback control system. In order to study this proposal, measurements of fractional sodium reabsorption were made prior to and 30 sec. - 20 min. following abrupt reductions or elevations in renal artery perfusion pressure. It was observed that the adjustment in proximal sodium reabsorption was fully established within the first 30 seconds of these maneuvers, an interval which is believed to be too brief to be consistent with a humoral adjustment. In these same experiments the change in reabsorptive rate and the simultaneous change in calculated cross sectional area of the tubule lumen were rarely proportional. In order to test the second major proposal to explain glomerulotubular balance, which presumes that the absolute rate of sodium reabsorption is governed by tubule volume, these same measurements were made prior to and during periods of elevated ureteral pressure. Despite large increments in calculated cross sectional area produced by raised ureteral pressure, the absolute rate of sodium reabsorption either remained relatively unchanged, or fell in proportion to the change in GFR (i.e. glomerulotubular balance was preserved). On the basis of these measurements, it is suggested that tubule geometry plays little role in the regulation of sodium reabsorption in the rat nephron.

Proposed Course of Project: Project is completed.

Honors and Awards: None

Publications: Brenner, B.M., Bennett, C.M. and Berliner, R. W.: The relationship between glomerular filtration rate and sodium reabsorption by the proximal tubule of the rat nephron. J. Clin. Invest. (in press).

Serial No. NHI -262

1. Kidney & Electrolyte Metabolism
2. Renal Mechanisms
3. Bethesda, Maryland

PHS - NIH
Individual Project Reports
July 1, 1967 through June 30, 1968

Project Title: The effect of dilution and expansion of the blood volume on proximal tubule sodium reabsorption in the dog.

Previous Serial Number: None

Principal Investigators: Franklyn G. Knox, M. D.
Fred S. Wright, M. D.
Stuart S. Howards, M. D.
Robert W. Berliner, M. D.

Cooperating Units: None

Man Years:
Total: 6/12
Professional: 6/12
Other: 0

Project Description:

Objectives: To evaluate the independent effects of dilution of the blood and expansion of the blood volume on sodium reabsorption by the proximal tubule and on sodium excretion in the urine.

Methods: Collections of urine, blood and proximal tubule fluid were made during hydropenia in 20 anesthetized dogs. The hematocrit of the dogs was then decreased without changing the blood volume by exchange circulation with a reservoir containing artificial plasma. Second samples of proximal tubule fluid were obtained from the previously punctured tubules at the original puncture sites and blood and urine samples were collected. The contents of the reservoir was infused and third samples of tubule fluid, blood and urine were obtained. In 8 experiments the blood volume was measured in hydropenia, dilution, and expansion phases. In 6 experiments the dog's blood was circulated through the

reservoir a second time instead of infusing the reservoir contents into the dog.

Major Findings: Fractional reabsorption by the proximal tubule was significantly decreased by dilution of the blood but sodium excretion was not significantly changed. Expansion of the blood volume with blood previously equilibrated with the dog resulted in a significant decrease in sodium reabsorption by the proximal tubule despite a decrease in renal blood flow. Fractional reabsorption by the proximal tubule did not change in the control experiments where the dog's blood was circulated through the reservoir a second time. It is concluded that both dilution of the blood and expansion of the blood volume can independently depress sodium reabsorption by the proximal tubule.

Proposed Course: Project completed.

Honors and Awards: None

Publications: Manuscript submitted to the Amer. J. Physiol.

724

Serial No. NHI - 263
1. Kidney & Electrolyte Metabolism
2. Renal Mechanisms
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of changes in extracellular fluid volume on sodium reabsorption by the proximal tubule of the rat

Previous Serial Number: None

Principal Investigators: Barry M. Brenner, M. D.
Fred S. Wright, M. D.
Robert Keimowitz, M. D.
Cleaves M. Bennett, M. D.
Robert W. Berliner, M. D.

Cooperating Units: None

Man Years:
Total: 24/12
Professional: 20/12
Other: 4/12

Project Description:

Objectives: To characterize the adjustment in sodium reabsorption by the proximal convoluted tubule of the rat following alterations in extracellular fluid volume (ECFV). ECFV expansion was produced in control antidiuretic animals by the administration of graded doses of isoncotic plasma and isotonic saline in quantities varying from 1.2 ml/hr to 24 ml/hr. ECFV contraction was accomplished by means of transperitoneal fluid movement in response to the intraperitoneal administration of polyethylene glycol (PEG) (MW 4000).

Methods: Sodium reabsorption by the proximal tubule was measured in two ways. In free flow studies, the concentration of inulin in tubule fluid relative to that in plasma provides a precise index of the extent of salt and water reabsorption in this segment of the nephron. Other experiments employed the shrinking drop technique, a method which provides an estimate of the rate of transepithelial fluid movement independent of changes in the rate of glomerular filtration or the velocity of linear flow.

In addition, by measuring the concentration of inulin and sodium in plasma and urine, it was possible to calculate GFR and fractional sodium excretion. Changes in plasma volume were examined by measuring changes in hematocrit.

Major Findings: The extent of fractional sodium reabsorption by the proximal convoluted tubule in the rat is influenced greatly by the volume of

ECF. Following a 12% contraction in plasma volume, fractional sodium reabsorption in the proximal tubule increases by an average of 20%. The increase of 20% represents the average change in fractional reabsorption of 20 tubules in 7 rats studied prior to and following and intraperitoneal administration of an isotonic solution of PEG. In contrast, graded degrees of expansion of ECFV associated with increments in plasma volume of 2%, 4% and 26% were associated with depression of fractional sodium reabsorption of 20%, 24% and 33% respectively. Administration of isoncotic plasma produced a 30% increase in plasma volume and a 20% decrease in fractional sodium reabsorption. Although ECFV expansion is associated regularly with depression of proximal sodium reabsorption, only when the magnitude of this depression exceeded 30% was there an appreciable increase in fractional sodium excretion.

Depression of sodium reabsorption by the proximal tubule after expansion of the ECFV was also shown by the shrinking drop method of Gertz. Absolute sodium reabsorption is decreased by infusions of plasma and of saline in quantities which expand the plasma volume.

It has been reported by others that a humoral factor present after ECFV expansion depresses proximal sodium reabsorption. Plasma from saline-loaded rats and dogs was infused and sodium reabsorption measured by the shrinking drop method. Plasma from saline-loaded animals did not have higher natriuretic activity than plasma from hydropenic animals.

In a further effort to measure natriuretic activity in plasma from saline-loaded animals, dialysates were used to form the shrinking drops. Dialysates of plasma from expanded animals as well as of plasma obtained during antidiuresis were prepared, using Visking tubing and Ringer's bicarbonate buffer. After 18 hours of dialysis at 4°C, the dialysates were randomized in double blind fashion and instilled into the lumen of proximal tubules using the split-droplet technique of Gertz. In 7 experiments, the mean half-time for reabsorption using the dialysate from natriuretic plasma was not significantly different from that derived from the plasma of non-expanded donor animals.

Proposed Course of Project:

1. Continue studies directed at validation of the work of Rector, et al. (J. Clin. Invest. 47: 761, 1968) regarding the presence of a humoral inhibitor of proximal sodium reabsorption.

2. Perform studies on the role of physical factors as they relate to the control of sodium reabsorption.

Honors and Awards: None

Publications: None

726

Serial No. NHI - 264
1. Kidney & Electrolyte Metabolism
2. Membrane Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of adrenergic agents on the response of the toad's urinary bladder to vasopressin.

Previous Serial Number: NHI-259.

Principal Investigators: Joseph S. Handler, M.D.
Jack Orloff, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 4/12
Professional: 4/12
Other: 0

Project Description:

Objectives: The urinary bladder of the toad responds to vasopressin with an increase in its permeability to water and in the rate of active sodium transport. The bladder responds similarly to adenosine 3',5'-phosphate (3',5'-AMP), the intracellular mediator of the action of the hormone and to theophylline which inhibits the degradation of 3',5'-AMP.

It has recently been reported that catecholamines, which are known to act on certain tissues (muscle, liver, brain, avian erythrocytes) by increasing their rate of production of 3',5'-AMP, may alter the rate of glucagon stimulated insulin secretion by the pancreas. Glucagon probably acts on pancreatic islet tissue by stimulating the production of 3',5'-AMP in the islet cells. This study is designed to evaluate the effect of catecholamines and other hormones on the response of the bladder to vasopressin.

Major Findings:

The water permeability response of the toad's urinary bladder to submaximal concentrations of vasopressin or theophylline is inhibited by 10^{-6} M epinephrine or norepinephrine. The catecholamines do not inhibit the submaximal response to 3',5'-AMP nor do they inhibit the maximal response to high concentrations of vasopressin. The inhibitory effects are blocked by α-adrenergic blocking agents (10^{-4} M phenoxybenzamine or 10^{-4} M phentolamine), but not by a β-adrenergic blocking agent (10^{-4} M propranolol). These results are interpreted as indicating that α-adrenergic activity decreases the production of 3',5'-AMP by inhibiting the enzyme adenyl cyclase. The effect of isoproterenol alone and in combination with α- and β-adrenergic blocking agents was highly variable, presumably because of seasonal factors. Although the results of these experiments are compatible with the interpretation that β-adrenergic activity stimulates adenyl cyclase activity, other interpretations have not been excluded. None of the agents examined affected the water permeability of the bladder in the absence of vasopressin, 3',5'-AMP or theophylline. The response to vasopressin was not altered by glucagon, ACTH, or serotonin. 10^{-5} M norepinephrine inhibited the short-circuit current response to low concentrations of vasopressin.

Proposed Course of Project: Project is completed.

Honors and Awards: None

Publications: Manuscript submitted to the Amer. J. Physiol.

128

Serial No. NHI - 265
1. Kidney & Electrolyte Metabolism
2. Membrane Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of aldosterone on the rate and control of glycolysis in the urinary bladder of the toad

Previous Serial Number: None

Principal Investigators: Joseph S. Handler, M. D.
Jack Orloff, M. D.

Cooperating Units: None

Man Years:
Total: 14/12
Professional: 2/12
Other: 12/12

Project Description:

Objectives: Work performed in a number of laboratories has established that the stimulation of sodium transport by the toad bladder in response to aldosterone depends upon the availability of suitable substrate and is associated with an increased rate of oxidation of these substrates. This study is concerned with the effect of aldosterone on the rate and control of glycolysis and the relationship of the effects on glycolysis to the stimulation of sodium transport by the hormone.

Major Findings: The protocol consists of incubating paired tissue from the same bladder overnight (16 hours) in Ringer's solution containing 20 mM glucose. 2×10^{-8} M aldosterone is added to the solution bathing the experimental bladder. The next morning the tissues are transferred to Ringer's solution containing 5 mM glucose at which time the rate of sodium transport is estimated by measuring the short-circuit current, and the rate of glycolysis is estimated by measuring the rate of lactate production and the rate of oxidation of glucose, uniformly labeled with carbon-14.

Under these conditions, tissue exposed to aldosterone has a sodium transport rate four times the rate in paired control tissue, and a glycolytic rate twice that in paired control tissue. If the tissues are made anaerobic after 16 hours, when the aldosterone effect on sodium transport is evident, the short-circuit current falls in both preparations, but remains three to four times greater in the aldosterone-treated tissue. Under anaerobic conditions, both preparations have a high rate of lactate production, and the rate of lactate production remains higher in the tissue treated with aldosterone. On the other hand, if sodium transport is inhibited by the addition of 10^{-4} M

729

ouabain (under aerobic conditions), the short-circuit current falls in both preparations to about the same value, and the rate of glycolysis falls in both preparations with the result that there is no longer a difference in any of these measurements between the aldosterone-treated and control preparations. Thus it would appear that the stimulation of glycolysis by aldosterone is coupled to the simultaneous stimulation of sodium transport.

The mechanism by which aldosterone controls the rate of glycolysis under aerobic conditions has been examined by measuring the concentration of glycolytic intermediates and some co-factors in tissue that has been extracted into perchloric acid after metabolic processes were stopped by freezing in liquid nitrogen. The concentration of glucose-6-P and fructose-6-P was lower and the concentration of fructose-1, 6-diP and triose-P was higher in aldosterone-treated than in control tissue, indicating activation of the enzyme phosphofructokinase. There was also evidence of activation of pyruvate kinase in that aldosterone caused a fall in the concentration of phosphoenolpyruvate and a rise in the concentration of pyruvate. The factors altering the activity of these enzymes were not elucidated in that there was no change in the concentration of ATP, ADP, 5'-AMP or inorganic phosphate. The concentrations of other co-factors and intermediates known to affect the activity of these enzymes have not been determined. There was a significant fall in the concentration of creatine-phosphate with a reciprocal rise in the concentration of creatine in tissue treated with aldosterone. This is probably the result of increased energy utilization as ATP by the increased sodium transport rate induced by aldosterone. The fact that the concentration of a high energy compound is lower in aldosterone-treated than in control tissue indicates that in hormone-treated tissue, energy production lags behind energy expenditure by sodium transport. Thus, the response to aldosterone must include a pathway that directly affects sodium transport.

Proposed Course of Project: Project is completed and manuscript is in preparation.

Honors and Awards: None

Publications: None

730

Serial No. NHI - 266
1. Kidney & Electrolyte Metabolism
2. Membrane Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of mucosal and of serosal hyperosmolality on water permeability of the urinary bladder of the toad

Previous Serial Number: None

Principal Investigators: Shigeharu Urakabe, M. D.
Joseph S. Handler, M. D.
Jack Orloff, M. D.

Cooperating Units: None

Man Years:
Total: 8/12
Professional: 8/12
Other: 0

Project Description:

Objectives: This study is an attempt to characterize the effects of hyperosmolality on water flux in toad bladder using urea and its related compounds, carbohydrates, and various salts.

Major Findings:

1. Hyperosmolality was achieved with urea and its related compounds (urea, methylurea, 1,3-dimethylurea, acetamide, thiourea, thioacetamide, and dimethylsulfoxide), carbohydrates (glycerol, mannitol, sucrose, and raffinose) and ionized salts (sodium chloride, potassium chloride and sodium isethionate).

Hypertonic urea, thiourea, methylurea and the three salts added to the mucosal side resulted in a remarkable increase in net water flux across the bladder. On the other hand, 1,3-dimethylurea, acetamide, thioacetamide, DMSO, glycerol and raffinose induced only a slight increase. No clear relationship between the permeance of the compounds studies and their effects was noted.

2. On the other hand, serosal hyperosmolality induced by those compounds which are permeant induced only slight increase of net water flux, while mannitol, sucrose, raffinose and sodium salts which do not penetrate readily resulted in marked increase in water permeability.

3. Unidirectional water permeability coefficient measured with tritiated water changed in a manner similar to the alterations in net flow.

4. In the case of hyperosmolality induced by urea or mannitol, so-called non-linear osmosis was demonstrated. In addition, a critical concentration for this effect was observed which was approximately 120 mM for both.

5. The effect of hyperosmolal urea on the mucosal surface continued at least for 60 minutes after the return of tissue to normal osmolality.

6. The above effects were noted in the absence of vasopressin. In the presence of vasopressin net water fluxes were in many cases not further changed. Also it was observed that the direction of water flux reversed 60 to 100 minutes after adding vasopressin in the case of urea, methylurea, 1,3-dimethylurea, acetamide and thioacetamide but did not reverse with thiourea, DMSO, carbohydrates, or the ionic compounds tested above.

From these results it is obvious that the effects of hyperosmolality on water permeability are different from compound to compound due to chemical structure, and that effects of mucosal or serosal hyperosmolality depend upon entirely different mechanisms.

7. In association with these studies analysis of changes in potential difference, short circuit current and resistance were determined.

Proposed Course of Project: Project is completed.

Honors and Awards: None

Publications: Manuscript in preparation.

Serial No. NHI - 267
1. Kidney & Electrolyte Metabolism
2. Membrane Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of adrenal steroid hormones on the permeability response of the toad's urinary bladder to vasopressin. A non-mineralocorticoid effect.

Previous Serial Number: None

Principal Investigators: Joseph S. Handler, M. D.
Shigeharu Urakabe, M. D.
Jack Orloff, M. D.

Cooperating Units: None

Man Years:
Total: 4/12
Professional: 4/12
Other: 0

Project Description:

Objectives: The urinary bladder of the toad has been employed by several laboratories to study the stimulatory effect of adrenal steroid hormones on sodium transport. Aldosterone is the prototype hormone eliciting this mineralocorticoid effect, and studies have ranged from characterization of a nuclear receptor for aldosterone to the demonstration that bladders that have responded to aldosterone manifest a greater increase in short-circuit current (sodium transport rate) in response to vasopressin than controls depleted of the effects of steroid hormones. This study is designed to examine the effect of adrenal steroid hormones on the water permeability response to vasopressin and the relationship of this effect to the mineralocorticoid effect of these hormones.

Major Findings: The general protocol consists of measuring the initial short-circuit current and the water or urea permeability response to vasopressin of paired tissue from the same bladder within a few hours of removal from the toad. The tissues are then incubated overnight in Ringer's solution containing abundant glucose with or without steroid hormone. In the absence of hormone, overnight incubation generally leads to a fall in short-circuit current (scc) to about 75 percent of the initial value, whereas incubation with high concentrations (10^{-7} M) of adrenal steroid hormones generally leads to a rise in scc to three times the initial value. When vasopressin is added after the overnight incubation the increment in scc is three to four times greater in steroid-treated bladders than in paired controls. When the water permeability response to vasopressin is tested after overnight incubation it is found that the

steroid-depleted tissues (controls) manifest about one-half the increase in permeability they did initially whereas tissue incubated with 10^{-7} M steroid hormones has the same responsiveness it did initially. The urea permeability response to vasopressin and the water permeability response to 3',5'-AMP, the intracellular mediator of the response to vasopressin, are also diminished by overnight incubation in vitro, but maintained at normal levels by adrenal steroid hormones. Thus adrenal steroid hormones, in addition to their previously recognized effect on scc and the scc response to vasopressin, maintain the permeability response of the toad bladder to vasopressin. This latter effect appears to be beyond the step at which 3',5'-AMP is made in response to vasopressin.

The steroid-induced pathway mediating the enhanced water permeability responsiveness to vasopressin appears to be different than that mediating the effect of the steroid hormones on scc. When glucocorticoid analogs of adrenal steroid hormones are used to elicit the enhanced water permeability response to vasopressin and the rise in scc (the classical mineralocorticoid effect), they appear to have a greater activity in the former system than aldosterone (the prototype mineralocorticoid hormone). In addition, under conditions in which the scc response to dexamethasone (a synthetic glucocorticoid type hormone) is inhibited by a spirolactone (an agent that inhibits the mineralocorticoid effect of adrenal steroid hormones, supposedly by competing for receptor sites), there is virtually no inhibition of the effect of dexamethasone on the water permeability response to vasopressin. These experiments are interpreted as demonstrating two separate receptor and effector systems in the toad bladder, one mediating the mineralocorticoid effect of adrenal steroid hormones on scc, the other mediating a non-mineralocorticoid effect, assayed in this study as the permeability response to vasopressin.

Proposed Course of Project: Suitable conditions will be sought to examine the question of whether or not the non-mineralocorticoid effect of adrenal steroid hormones depends on a pathway involving protein synthesis, as has been suggested for the mineralocorticoid effect of these hormones.

Honors and Awards: None

Publications: None

734

Serial No. NHI -268
1. Kidney & Electrolyte Metabolism
2. Membrane Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of inhibitors on sodium flux and the sodium flux response to cyclic 3',5'-AMP in the urinary bladder of the toad.

Previous Serial Number: None

Principal Investigators: Stanley A. Mendoza, M. D.
Joseph S. Handler, M. D.
Jack Orloff, M. D.

Cooperating Units: None

Man Years:
Total: 10/12
Professional: 10/12
Other: 0

Project Description:

Objectives: Active transport of sodium from the mucosal bathing medium to the serosal bathing medium by the toad bladder is well known. The net transport of sodium is augmented by vasopressin and by theophylline (both of which act by increasing the intracellular concentration of cyclic 3',5'-AMP) and by exogenous cyclic 3',5'-AMP. Attempts to study the mechanism of sodium transport in the toad bladder have implicitly assumed that baseline transport and transport stimulated by one of these agents occur through the same pathway. This assumption has been tested by studying the effects of a variety of inhibitors of sodium transport (chlorpropamide, a sulfonylurea compound; fluoroacetate, an inhibitor of the citric acid cycle; L589,420-0-2, an ethacrynic acid analog which blocks sulfhydryl groups without affecting Na-K activated ATPase; and ouabain, a Na-K ATPase inhibitor).

Major Findings: A concentration of each inhibitor was found which lowered baseline short-circuit current and net sodium flux

about 40-60%. After the sodium flux reached a new steady-state level, cyclic 3',5'-AMP was added. In ouabain-treated bladders, the response to cyclic 3',5'-AMP was inhibited about 50%. That is, the inhibition of the response to cyclic 3',5'-AMP was proportional to the inhibition of baseline sodium transport. The other three inhibitors were quite different. The response to cyclic 3',5'-AMP was normal in bladders whose baseline sodium transport was markedly inhibited by pretreatment with any of these inhibitors.

Since it is possible to find agents which inhibit baseline sodium flux without altering the response to cyclic 3',5'-AMP, it is concluded that control sodium flux and sodium flux stimulated by cyclic 3',5'-AMP may occur through different pathways.

Proposed Course of Project: Project has been completed.

Honors and Awards: None

Publications: Manuscript is in preparation.

Serial No. NHI - 269
1. Kidney & Electrolyte Metabolism
2. Membrane Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of amphotericin B on sodium transport in the toad's urinary bladder.

Previous Serial Number: NHI - 263

Principal Investigators: Stanley A. Mendoza, M. D.
Joseph S. Handler, M. D.
Jack Orloff, M. D.

Cooperating Units: None

Man Years:
Total: 4/12
Professional: 4/12
Other: 0

Project Description:

Objectives: It has been found in this laboratory and elsewhere amphotericin B causes little or no increase in short-circuit current on the day the bladder is removed from the toad, but elicits a marked increase in short-circuit current after the bladder has been incubated overnight. In order to characterize the mechanism by which amphotericin B increases short-circuit current and net sodium flux, the effect of a variety of inhibitors on this process was studied.

Major Findings: On the day of removal from the toad, amphotericin B caused no increase in short-circuit current in the fall of the year and a small increase in the spring and summer. During this latter period, bi-directional sodium fluxes were performed and the increase in short-circuit current was found to be due to increased net sodium flux. Pretreatment with ouabain caused the short-circuit current and net sodium flux to fall to near 0. The addition of amphotericin B at this point caused a rise in short-circuit current equal to that elicited by

737

amphotericin B in paired controls. Unexpectedly, net sodium flux, which rose when amphotericin B was added to controls, actually fell when the drug was added to ouabain-treated bladders. Dinitrophenol-treated bladders acted similarly. In view of the discrepancy between the short-circuit current and net sodium flux, it was felt that amphotericin B is not a suitable agent to use in the study of sodium transport in the toad bladder.

Proposed Course of Project: The study has been put aside temporarily.

Honors and Awards: None

Publications: None

Serial No. NHI - 270
1. Kidney & Electrolyte Metabolism
2. Membrane Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Observations on lipid metabolism of the toad bladder.

Previous Serial Number: None

Principal Investigators: Douglas R. Ferguson, M.D.
Joseph S. Handler, M. D.
Jack Orloff, M. D.

Other Investigators: Dr. Martha Vaughan

Cooperating Units: Laboratory of Metabolism, NHI

Man Years:
Total: 13/12
Professional: 13/12
Other: 0

Project Description:

Objectives: This study is designed to investigate the lipid content and metabolism of toad bladder epithelial cells, and the changes which may occur when the water permeability and rate of active sodium transport are altered by vasopressin.

Methods: Experiments have been carried out on the isolated bladder of the toad Bufo marinus. They have involved the incubation of bladders with radioactive palmitate, the collection of $^{14}CO_2$, and the extraction of lipids from bladder mucosal cells. The lipids were separated by thin layer chromatography on silica gel. They were then measured by chemical methods or their isotope content estimated by liquid scintillation counting.

Major Findings:

1. Palmitic acid is oxidized to CO_2 by the toad bladder. The rate of oxidation of palmitate is increased by vasopressin

only in the presence of active sodium transport. The rate of oxidation of palmitate is diminished when the rate of sodium transport is diminished by ouabain, indicating that in these experiments oxidation of the fatty acid is partially coupled to sodium transport. Palmitate oxidation is also increased by the presence of glucose in the Ringer's solution.

2. Palmitic acid is incorporated into lyso-lecithin, phosphatidylethanolamine and lecithin, and probably also into phosphatidyl serine and phosphatidyl inositol. The rate of incorporation of palmitate into lecithin is greatest. Palmitic acid is also incorporated into triglycerides and cholesterol esters.

Proposed Course of Project: Attempts are being made to separate mucosal cells from toad bladders by incubation with collagenase. The rate of oxidation of palmitate by the isolated cells with and without vasopressin is being examined. It is hoped to obtain more accurate estimates of the lipid content of these cells, and possible changes induced by vasopressin.

Honors and Awards: None

Publications: None

Serial No. NHI -271

1. Kidney & Electrolyte Metabolism
2. Membrane Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of chlorpropamide on the osmotic flow of water in the toad's urinary bladder.

Previous Serial Number: None

Principal Investigator: Stanley A. Mendoza, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 2/12
Professional: 2/12
Other: 0

Project Description:

Objectives: Chlorpropamide, a sulfonylurea derivative, is known to be effective in the therapy of patients with hypothalamic and pituitary diabetes insipidus but not in patients with nephrogenic diabetes insipidus. It has been proposed that chlorpropamide interacts with vasopressin receptors in the kidney. It was decided to study the effect of this drug on the osmotic flow of water in the urinary bladder of the toad, a tissue in which certain aspects of the response to vasopressin have been studied.

Major Findings: Chlorpropamide alone had no effect on the osmotic flow of water in the toad bladder. The drug did potentiate the effect of vasopressin and theophylline, both of which increase the osmotic permeability of the toad bladder by increasing the intracellular concentration of cyclic 3',5'-AMP. Unexpectedly, the response of the toad bladder to exogenous cyclic 3',5'-AMP was inhibited by chlorpropamide. Therefore, the mechanism of action of chlorpropamide remains obscure. Nevertheless, it is

741

clear that under certain conditions the drug does alter the permeability of vasopressin-sensitive tissues.

Proposed Course of Project: Project has been completed.

Honors and Awards: None

Publications: Manuscript submitted to Endocrinology.

Serial No. NHI - 272
1. Kidney & Electrolyte Metabolism
2. Electrolyte Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Structural elements on the surface of the human erythrocyte ghost using a negative staining technique

Previous Serial Number: None

Principal Investigators: Alan S. Rosenthal, M. D.
Floyd M. Kregenow, M. D.

Cooperating Units: None

Man Years:
Total: 3/12
Professional: 3/12
Other: 0

Project Description:

Objectives: To delineate structural elements on the surface of the human erythrocyte ghost using a negative staining technique.

Methods: Human erythrocyte Hgb-free ghosts were prepared as described previously. The ghosts were air dried on the surface of formvar grids which were carbonized on the under surface. This process results in ghost disruption and often the exposure of a single membrane to the electron beam. The membranes were negatively stained with 1% uranyl formate or acetate.

Results: With the above procedure, numerous previously unidentified structures are presented on the negatively stained erythrocyte ghost. The most striking are "tunnel like" spiraled structures numbering between 100-1000/ghost. In addition, dumbbell and doughnut shaped structures are occasionally seen. These structures are present on Hgb-free ghosts prepared by the method of Dodge et al. and on ghosts prepared using a modification of this method which utilizes EDTA. Although these structures are discreet morphologically rather than physically or physiologically, their significance remains unclear.

Proposed Course of Project: To investigate the physical and possible physiological significance of these structures.

Honors and Awards: None

Publications: None

743

Serial No. NHI - 273
1. Kidney & Electrolyte Metabolism
2. Electrolyte Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: To investigate the protein composition of Fractions I and II, and to compare these fractions from the red blood cells of other species.

Previous Serial Number: None

Principal Investigator: Dr. Floyd M. Kregenow

Other Investigator: Dr. Leroy Hood

Cooperating Unit: Immunology Branch, National Cancer Institute

Man Years:
Total 5/12
Professional: 5/12
Other: 0

Project Description:

Objectives:

1. To determine the homogeneity of the two fractions by means of electrophoresis, ultracentrifugation and end group analysis.

2. To analyze the components of these two fractions from the red blood cells of other species.

Methods: The two fractions were obtained from human red blood cells using the previously described techniques. Dog and high K sheep red blood cells were collected in 4% sodium citrate solution. Fraction I was prepared in the usual manner from their Hgb-free ghosts. Ultracentrifuge studies were performed with a Beckman Model E. Ultracentrifuge using schlierin optics. The fractions were electrophoresed on a modified discontinuous acrylamide system, 8 M starch gel, and the continuous acrylamide system

of Maizels. N Terminal amino acids were determined by the Stark method. Amino acid analysis was performed on a Beckman 120C amino acid analyzer and by the Formic acid paper electrophoretic system of Dwyer. The Fractions were reduced and aminoethylated as described by Hood.

Major Findings:

1. When crude EDTA or ATP Fraction I from human red blood cells is electrophoresed on modified discontinuous electrophoretic system, two bands appear with RF values $>$ .9, the fastest moving band trailing just behind the tracking dye which in this case is phenol red. The acrylamide system has been modified so that it 1) has a 4% porosity, 2) has been polymized by light after the addition of riboflavin, 3) has less than 3 mM TrisCl, and 4) has a lower gel Ph prior to electrophoresis of 8.1. Two peaks are obtained in the ultracentrifuge if the fractions are centrifuged in a buffer system with a similar electrolyte composition: 50 mM glycine Tris, 3 mM Tris Cl, and either .5 mM ATP or EDTA, Ph 8.1.

2. When the EDTA Fraction I is electrophoresed on 8 mM urea starch gel, when the buffer system is 50 mM glycine Tris, .5 mM EDTA, Ph 7.4, and then stained with Amido Schwarz, a main band appears as well as several minor ones. Ultracentrifugal analysis of this Fraction in 8 M urea using the identical buffer demonstrates a major symmetrical peak as well as a minor asymmetrical one.

An electrophoretic component similar to the major band can be obtained from Hgb-free ghosts of human RBC by dialyzing them at low salt concentrations against .1 M mercaptoethanol at 4° C for 18 hours or by suspending them in 8 M urea + .1 M mercaptoethanol at room temperature at higher salt concentrations.

3. A similar electrophoretic band on 8 M urea starch gels is obtained from dog and high K sheep when the standard procedures are applied to their Hgb-free ghosts.

4. A partial purification of Fraction I can be accomplished on a Sepharose 4B column. The void volume contains both the fiber system and ATPase activity previously described. The heterogeneity of this fraction which is proteinaceous in nature

74

can be demonstrated by N Terminal analysis and electrophoresis on the continuous acrylamide system of Maizels. After the preparation has been first reduced and aminoethylated in the presence of 1% SDS, N Terminal analysis reveals 62% aspartic acid, and 23% glutamic acid, 4.3% glycine, 6.9% alanine, and 4% leucine. Electrophoresis of the preparation in the presence of SDS and 8 M urea reveals the presence of at least 5 main bands and several minor ones.

5. The heterogeneity of crude Fraction II can be demonstrated when this fraction is reduced and aminoethylated and electrophoresed on a similar system.

Proposed Course of Project: Project has been set aside temporarily.

Honors and Awards: None

Publications: Manuscript in preparation.

Serial No. NHI - 274

1. Kidney & Electrolyte Metabolism
2. Electrolyte Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The solubilization of a protein fraction from the human erythrocyte membrane and a characterization of the functional properties of this fraction and the remaining membrane elements.

Previous Serial Number: None

Principal Investigators: Floyd M. Kregenow, M.D.
Alan S. Rosenthal, M.D.

Cooperating Units: None

Man Years:
Total: 18/12
Professional: 9/12
Other: 9/12

Project Description:

Objectives:

1. To solubilize erythrocyte membrane proteins by a method which will allow the functional characterization of the solubilized fraction as well as the remaining membrane elements.

2. To characterize the functional properties of the solubilized protein fraction and to identify and purify the responsible elements.

3. To characterize the remaining membrane elements (vesicles).

Methods: Human red blood cells were collected from healthy Caucasian volunteers into a 4% sodium citrate solution. After removal of the buffy coat they were washed 4 times in 153 mM NaCl 17 mM KPO_4. Hgb-free ghosts were prepared by a modification of

the method of Dodge, Mitchell and Hanahan. The solubilized protein fraction was obtained from Hgb-free ghosts using the procedure described in major findings. The vesicles, prior to their functional characterization, were washed free of the supernatant fraction. Comparative functional studies were performed on washed vesicles and an equal aliquot of original intact ghosts.

Protein was determined by the method of Lowry, Biuret or 280 mμ transmittance using a Beckman or Zeiss spectrophotometer. Lipid was determined gravimetrically using a modification of the method of Reed and Weed. Sialic acid was determined by using the method of Warren, et al. Hexose was obtained by using the phenol sulfuric acid method. Carboxy C^{14} inulin space was evaluated by the method of Finn and Handler. ATPase activity was determined by the release of gamma phosphate 32 from ATP^{32} or by the release of inorganic phosphate from ATP by a modification of the method of Berenblum and Chain. A Leitz binocular microscope was used in the phase and dark field studies. Protein and membranes were negatively stained with 1% uranyl formate and examined using a Phillips EM - 200 electron microscope.

Major Findings: To obtain reconstituted ghosts which contain many of the normal membrane characteristics, osmotic shock must occur at low temperature, in the presence of divalent cation and with a certain minimal concentration of monovalent cations.

When these conditions are altered and osmotic shock is conducted at room temperature in the presence of a chelating agent such as .5 mM EDTA or .5 mM ATP and with less than 3 mM monovalent cations present, one obtains two gross fractions: (1) a soluble fraction containing mostly protein and (2) a particulate vesicular membrane fraction.

In observing the subdivision of the ghost membrane under the phase microscope, a phenomenon was observed which suggests that a membrane element is capable of contraction. Shortly after the solubilization procedures are applied (tonicity is lowered uniformly by dilution) the ghost population within 15-20 minutes subdivides into a structure (diameter 2-3 times that of the normal ghost) which consists of a radial array of finger-like vesicular projections. The addition of salt at this stage results in a retraction of the finger-like projections and contraction of the ghost. The divalent cations are more effective in producing this

response than the monovalent cations. Hypotonic solutions of divalent cations if they contain sufficient quantity of divalent cation to exceed the sequestering ability of the chelating agent (a quantity which would raise the extracellular osmotic gradient less than 5%) will also produce the response.

If the solubilization conditions are prolonged (using EDTA as the chelating agent) the membrane subdivides to form a preparation which contains isolated vesicles and chains of vesicles. This preparation aggregates if the solubilization procedures are reversed provided some of the original supernatant fraction is present. Clumping can be shown to be dependent on a component in the supernatant fraction and during clumping protein is removed from this fraction. Dispersion of the clumps occurs only when all three of the original solubilization procedures are reapplied. Clumping has many similarities to the contraction phenomenon and may be an abortive form of this phenomenon.

The washed free floating vesicles have many of the properties of intact ghosts. Each red cell fragments into approximately 150 vesicles which have an average diameter of 1 μ. They contain approximately 90% of the lipid, 96% of the sialic acid, and 92% of the hexose, and 75% of the protein present on the original intact ghost. Electron microscopy demonstrates a unit membrane appearance. They behave as osmometers which indicates the vesicular membrane has a low cation permeability relative to water permeability. Experiments with C^{14} inulin indicate that the osmotic response occurs in a non-inulin space (intravesicular) of 40-60%. They are capable of producing lactic acid from fructose diphosphate when the appropriate co-factors are added. In addition, they possess approximately 50% of their original ouabain sensitive ATPase activity.

There does appear to be a selective loss of at least two membrane constituents: (1) the first is a ouabain insensitive ATPase activity, and (2) the second constituent is a fiber appearing system or systems which is demonstrable on dried ghosts negatively stained with 1% uranyl formate.

The crude supernatant fraction forms a gel when salt is added. The gel has elastic properties. Once again the divalent cations produce gelling at a lower concentration than the monovalent cations. Negative stained preparations of the salt-treated crude

fraction with uranyl formate demonstrates a fiber system or systems which resemble that on the intact ghost membrane and which is missing from the vesicles. The crude supernatant fraction also has ouabain insensitive ATPase activity which is both Mg^{++} and Ca^{++} dependent.

A partial purification of this supernatant fraction can be accomplished on a Sephrose 4B column equilibrated with an isotonic solution containing physiological concentrations of Ca^{++} and Mg^{++}. The fiber system separates with the void volume and is associated with ouabain insensitive ATPase activity. The void volume contains almost entirely protein. There is less than .1% hexose or sialic acid and less than 1% lipid when compared on a weight basis to the quantity of protein present.

Proposed Course of Project: Project is completed.

Honors and Awards: None

Publications: Manuscript in preparation.

Serial No. NHI - 275
1. Kidney & Electrolyte Metabolism
2. Electrolyte Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The isolation and characterization of ouabain sensitive and ouabain insensitive ATPase activity from the human erythrocyte membrane.

Previous Serial Number: None

Principal Investigators: Floyd M. Kregenow, M.D.
Alan S. Rosenthal, M.D.

Cooperating Units: None

Man Years:
Total: 8/12
Professional: 5/12
Other: 3/12

Project Description:

Objectives:

1. To characterize the ouabain insensitive ATPase activity present in the previously solubilized EDTA supernatant fraction. (Fraction I)

2. To devise a method for removing ouabain sensitive ATPase activity from the washed vesicles. Such a method would take advantage of the physiological characteristics of the vesicles and would result in a step-wise isolation of functional membrane parameters.

3. To characterize the ouabain sensitive ATPase activity. (Fraction II)

Methods: The methods are similar to those previously described. Ouabain insensitive ATPase activity was characterized in the EDTA prepared supernatant fraction (Fraction I). The remaining vesicles were washed free of their fraction and then

subjected to an extraction procedure which will be described in the major findings. This second procedure results in a preparation with ouabain sensitive ATPase activity. P^{32} was counted on a Tri Carb automatic scintillation counter.

Major Findings:

1. Fraction I contains a small quantity of divalent cation insensitive ATPase activity and a much greater quantity of ATPase activity which is dependent on the presence of either Ca or Mg. Ca^{++} dependent activity which is the largest is inhibited by increasing concentrations of Mg^{++}. Divalent cation activity is not inhibited by ouabain 10^{-4} M in the absence of Na^{+} and K^{+}.

2. In isolating Fraction II, advantage was taken of the fact that the vesicles appear to contain only ouabain sensitive ATPase activity. If washed vesicles are suspended in a medium containing 50% glycerol, 2 M $MgCl_2$, 2 mM cysteine, 1 mM ATP, and physiological concentrations of Na and K at -20° C and then centrifuged at 150,000 g for 5 hours at 0° C, a clear lower fraction is obtained. After removing the glycerol and excess Mg from the lower clear fraction by dialysis against a solution containing physiological concentrations of Mg, Na and K, 2 mM cysteine, and 1 mM ATP, a preparation is obtained which consists of a small quantity of a white ppt and a much larger soluble fraction. This preparation contains < 20% of the vesicular protein as well as ouabain sensitive ATPase activity.

3. Preliminary experiments indicate that ouabain sensitive ATP activity is dependent on both the soluble fraction and ppt. Ouabain sensitivity is labile and affected by the ADP and ATP concentration, monovalent cations, and the length of incubation.

Proposed Course of Project:

1. To further characterize the ATPase activity in Fraction I and II.

2. To identify the component or components which are necessary for the activity.

3. To attempt to identify the ouabain's binding component in Fraction II using tritiated ouabain.

752

Honors and Awards: None

Publications: Manuscript in preparation.

Serial No. NHI - 276
1. Kidney & Electrolyte Metabolism
2. Electrolyte Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Response of nucleated RBC to variations in the tonicity of the extracellular media.

Previous Serial Number: None

Principal Investigators: Daniel H. Riddick, M. D.
Floyd M. Kregenow, M. D.

Cooperating Units: None

Man Years:
Total: 6/12
Professional: 6/12
Other: None

Project Description:

Objectives:

1. To study the response of a nucleated RBC to changes in the tonicity of the extracellular media.

2. To characterize the membrane mechanism which governs the cation movements associated with this pertubation.

Methods: Cell volume is calculated from a gravimetric determination of cell water content. Na^+ and K^+ concentration are determined on a Baird flame photometer. From a knowledge of changes in both these parameters and the hematocrit net cation changes are calculated for both the extracellular and intracellular compartments.

Unidirectional Na^{24} and K^{42} flux measurements are obtained by using previously described techniques.

Chloride ratios were determined on a Cotlove Chloridometer.

Major Findings:

Volume response.

Duck red blood cells placed in plasma which has been made hypertonic by the addition of 50 mOsmole KCl shrink instantaneously and over a one-half to 1-hour period recover their original volume and then maintain it. During the recovery stage there is a net accumulation of K from the plasma against a concentration gradient. It is the osmotic activity of this additional cation which is presumably responsible for the recovery stage. During the recovery stage there is a small but significant loss of intracellular Na. The control K^{42} influx is 9 mM/L cells /hr. Cells shrunken in KCl and thus suspended in a K^{+} concentration five times normal have a 10-fold increase in unidirectional K^{42} influx during the recovery period and also 1 hour later after a new steady state has been reached.

Duck red blood cells placed in plasma whose osmolality has been decreased 50 mOsmols/L by the addition of demineralized water swell instantaneously and over a 1-hour period recover their original volume and then maintain it. During the recovery phase there is a loss of Na and K from the cell in approximately equal quantities. The loss of these osmotically active agents is presumably responsible for the shrinking that occurs in the recovery phase. Cells swollen in hypotonic plasma and thus suspended in less K than the control cells have a slightly diminished K influx during the recovery phase. The K^{42} influx of these cells 1 hour later after they have reached a new steady state is increased 3-fold over normal despite the fact the K concentration is now near the normal value.

None of the altered K influx determinations at present appear to be influenced by 10^{-4} M ouabain.

Proposed Course of Project:

1. To define more clearly the cation movements associated with this osmotic phenomenon.

2. To define more precisely the membrane mechanism or mechanisms responsible for this volume regulation.

755

3. To investigate the metabolic and extracellular factors necessary for the observed changes.

Honors and Awards: None

Publications: None

Serial No. NHI - 277
1. Kidney & Electrolyte Metabolism
2. Electrolyte Transport
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Formation and function of the contractile vacuole.

Previous Serial Number: None

Principal Investigator: Daniel H. Riddick, M. D.

Cooperating Units: None

Man Years:
Total: 6/12
Professional: 6/12
Other: 0

Project Description:

Objectives:

1. To investigate the nature of formation of the contractile vacuole.

2. To define the solution contained within the vacuole.

3. To investigate the function of this organelle.

Methods: The principal method of attack was classical micropuncture. Osmolality and Na^+ and K^+ concentration of the resulting sample of from .02-.1 nl volume were determined on the Ramsey-Brown osmometer and helium glow spectrophotometer, respectively.

Vacuoles were isolated from the amoeba by suction pipette. The vacuoles were transferred to solutions of different ionic and osmotic components and the behavior of the vacuole was noted.

Major Findings:

1. Vacuole volume determination: Volumes of as small as

757

.01 nl can be accurately determined to ± 2%.

2. Osmolality determination: The osmolality was measured on 10 samples of cytoplasm and contractile vacuoles. The cytoplasm has an osmolality 117 mOsm/L with SE = ± 2.5. The vacuole osmolality is 51 mOsm with SE = ± 1.6. The osmolality of the bathing solution was < 2 mOsm/L. The ratio of the osmolality of the vacuole to the cytoplasm was fairly constant at .493 with SE = .018. There was no correlation between vacuole volume and osmolality.

3. $[Na^+]$ and $[K^+]$ Analyses: The determinations were quite difficult technically and therefore only four determinations were made. The average Na^+ and K^+ concentrations of the contractile vacuole were 19.9 mM/L and 4.6 mM/L respectively with a range of 18.8-20.3 mM/L and 4-5.1 mM/L respectively. There was no correlation between ion concentration and vacuole volume.

The average Na and K concentrations of the cytoplasm were .57 mM/L and 31 mM/L with a range of .51-.73 mM/L and 29-33 mM/L respectively.

4. Vacuole Growth: Increase in vacuole volume with time appears to be primarily by fusion of the minute vesicles surrounding the growing vacuole and by occasional fusion of small vacuoles with one another.

5. Isolated vacuole: The isolated contractile vacuole behaves as an osmometer, i.e., it retains at least some of its selective membrane properties, but does cease its increase in size and in fact shrinks slowly in a 30-minute period when isolated in medium similar to the cytoplasm.

Proposed Course of Project: The above constitutes all that is presently planned as regards this problem.

Honors and Awards: None

Publications: Riddick, Daniel H.: The contractile vacuole in the amoeba, Pelomyxa carolinensis. Amer. J. Physiol. - In press.

758

Serial No. NHI - 278
1. Kidney & Electrolyte Metabolism
2. Experimental Cardiovascular Diseases
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immune mechanisms in glomerulonephritis

Previous Serial Number: NHI - 264

Principal Investigators: Edward J. Leonard, M. D.
Philip Askenase, M. D.

Other Investigators: None

Cooperating Units: Arthritis and Rheumatism Branch
National Institute of Arthritis and Metabolic Diseases

Man Years:
Total: 24/12
Professional: 24/12
Others: 0

Project Description:

Objectives: Among the immune mechanisms that may play a role in the pathogenesis of glomerulonephritis which were outlined in last year's report, most of this year's work has been directed toward detection of circulating soluble immune complexes.

Methods: The method, as outlined last year, is based on the assumption that soluble immune complexes capable of causing inflammation should have activated the complement system and should have incorporated C'3 (the third component of complement) into the soluble molecular complex. Such a complex would have a molecular weight of at least 500,000. If plasma containing these large complexes is fractionated on G-200 Sephadex, the complex should be found in the first peak of protein to be eluted. This peak contains high molecular weight proteins which are excluded entirely by the gel. The normally occurring components of the immune complex, namely C'3 (or beta-1-c) and the antibody molecule IgG have molecular weights of the order of 150,000. Thus, unless they are combined in an immune complex or aggregated in some other way, they would not be in the G-200 exclusion volume but would be eluted in the second peak with the other globulins of their size range. Last year it was reported that C'3 was not detected in the G-200 exclusion volume of normal plasma, that it was found there if immune complexes were added to normal plasma before G-200 elution, and that it was not found there in 6 cases of lupus nephritis. C'3 was detected by a standard precipitin-in-gel method (Ouchterlony) in which the G-200 eluates were reacted with an antiserum to C'3. The minimum C'3 detectable by this method was about 25 micrograms.

759

There are no data on the minimum quantity of circulating complex required to cause glomerulonephritis in experimental animals. The production of chronic glomerulonephritis in rabbits by daily injections of antigen involved milligram quantities of circulating complex. On the other hand, the slow development of autoimmune glomerulonephritis in rats by the method of Heymann, recently studied by Edgington and Dixon, may occur with the order of nanogram amounts of circulating complex. For this reason, and in the light of the preliminary negative results with our first six cases of lupus nephritis, it was decided to develop a sensitive radioimmunoassay for immune complexes in human plasma. The work has had four facets: isolation of pure C'3 for both iodination and production of antibody, development of a solid phase radioimmunoassay with these reagents, improving resolution of plasma fractionation on G-200 Sephadex, and assay for the immune globulin moiety of the soluble complex.

Isolation of C'3. This was done by modification of published methods, which involve precipitation of C'3 as a euglobulin, followed by fractionation on DEAE and hydroxyl apatite columns. "Purity" of a protein isolated from a complex mixture is a relative term which depends on the sensitivity of methods used to assay for contaminants. In publications in which a single precipitin line on immunoelectrophoresis is used as a criterion of purity it is rare to find the concentration of the preparation stated. In our own case we have C'3 fractions which show only one precipitin line at concentrations of 1 mg/ml, but which show two or three faint contaminating lines at 15 mg/ml. For purposes of the radioimmunoassay the important requirement is that the C'3 preparation be free of any plasma component which would normally occur in the first peak of G-200 eluates. Therefore, our last step of C'3 isolation was chromatography on G-200 Sephadex, from which a second peak fraction was collected for iodination with I^{125}.

Solid Phase Radioimmunoassay. Radioactive antigen is mixed with a limited amount of antibody, which results in formation of antigen-antibody complex (Ag* Ab). Unlabelled antigen will compete with labelled antigen for the limited number of antibody sites, so that the more unlabelled antigen in the system the less label will be found in the complex. The most difficult step in radioimmunoassay is separation of free Ag* from Ag* bound to antibody (Ag* Ab). Recently Catt reported that sufficient antibody can be adsorbed to a solid surface for performance of radioimmunoassay. We have applied his approach successfully for radioimmunoassay of C'3. Briefly, an appropriate dilution of antiserum is added to a series of regular Packard gamma counting tubes. After equilibration, the antiserum is removed and tubes are rinsed. There is now a certain amount of antibody adsorbed to the tube surface. At the concentrations employed it appears that probably any protein becomes adsorbed non-specifically to foreign surfaces. Thus we have found that at concentrations of 1 microgram/ml, 30-40% of protein added becomes adsorbed to the tube. Therefore, after the tube is coated with antibody, it is equilibrated with relatively high concentrations of purified bovine serum albumin to prevent non-specific adsorption of C'3 antigen. In the next step, a solution containing a known amount of radioactively labelled C'3 is added; a standard curve is obtained by having a series of tubes with label plus increasing amounts of unlabelled antigen. After equilibration the solutions are removed, tubes are

rinsed and counted for adsorbed radioactivity. Controls and criteria for specificity are beyond scope of this report, but have been worked out and appear satisfactory. The minimum C'3 detectable is between 10 and 20 nanograms per sample, a 1000-fold increase in sensitivity over the precipitin-in-gel method.

Improvement of resolution on G-200. The concentration of C'3 in human plasma is about 1 mg/ml. C'3 not bound in a soluble complex should be eluted in the second peak of G-200 eluates. If the assay can detect 10 nanograms and 0.1 ml plasma is applied to the column, contamination of first peak by 1/10,000 of the second peak C'3 would be measurable as a baseline occurring in normal plasma. An effort has been made to use G-200 as an analytical tool, by employing G-200 Superfine in a long column with a small volume of applied sample. Conditions of application, flow and collection are carefully controlled. It is evident that resolution has been greatly improved. However, there is detectable C'3 in the first peak from normal plasma, probably at the 10-30 nanogram level. The significance of this is being evaluated at the present time.

IgG Fc assay. The circulating immune complex should be composed of an unknown antigen, antibody and complement components. The above discussion centered on detection of C'3. A parallel assay is being worked out for the antibody. There is no problem here except that IgG, a second peak protein, has antigenic determinants in common with IgM (macroglobulin), a first peak protein. However, there is an antigenically specific portion of IgG, the Fc fragment, which can be obtained by enzymatic digestion of IgG. It is distinct from IgM. A radioimmunoassay using IgG Fc* and specific antibody should thus provide us with a second assay for first peak immune complex.

Proposed Course of Project: There seems to be no question that milligram quantities of a variety of soluble complexes can cause acute or chronic glomerulonephritis in experimental animals. It is unlikely that such large amounts occur in human disease. We have nearly completed methods for detecting nanogram quantities of circulating soluble immune complexes in human plasma. We should now be in the position to evaluate claims about occurrence and pathogenicity of immune complexes. Do they occur at nanogram levels in normals? Are they found in response to infection or to immunization? Are they peculiar to or increased in vasculitis, nephritis or other conditions in which circulating autoantibodies are suspected? Does their presence correlate with, for example, active nephritis, or will it turn out that the soluble complex is a necessary but not sufficient condition for the development of this disease? Evaluation of appropriate clinical material is planned for the coming year with the above questions in mind.

Honors and Awards: None

Publications: Hajdu, S., Maximin, T. J. and Leonard, E. J.: Cardioglobulin: Separation, characterization, and assay of the individual components. Circulation Research XXII: 517-526, April 1968.

Leonard, E. J., Maximin, T. J. and Hajdu, S.: Cardioglobulin: Tissue localization and plasma activity with special reference to cardiovascular disease and lupus erythematosus. Circulation Research XXII: 527-536, April 1968.

Serial No. NHI - 279
1. Kidney & Electrolyte Metabolism
2. Experimental Cardiovascular Diseases
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of the Woodworth staircase phenomenon in heart and skeletal muscle

Previous Serial Number: NHI - 267

Principal Investigator: Stephen Hajdu, M. D.

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 12/12
Professional: 12/12
Others: 0

Project Description:

Objectives: The contractile force of mammalian cardiac tissue with the exception of the ventricular muscle of the guinea pig is kept up by two processes, the effect of which is distributed over the whole frequency range. In other words the well-known Bowditch staircase phenomenon sustains contractility at high frequency of stimulation and the so-called Woodworth (or reversed staircase phenomenon) at low frequencies. These two phenomena complement each other to the extent that the force of contraction of most of the mammalian myocardium becomes almost independent from the heart rate. While some knowledge has been accumulated on the cellular mechanism of the Bowditch phenomenon, very little is known on this point concerning the Woodworth phenomenon with the following exceptions: A possible connection between the cardioglobulins and the Woodworth phenomenon was suspected when it was found that the cardiotonic activity of the system could be demonstrated on cardiac tissue devoid of the Woodworth phenomenon, either naturally, as in frog and guinea pig ventricle or freed from it by the use of Ryanodine or long perfusion with artificial saline. A further connection between the cardioglobulins and the Woodworth phenomenon was observed when the sudden elimination of the Woodworth phenomenon by Ryanodine yielded a small but rather consistent release of Ca^{++} (measured as Ca^{45}) from left ventricle strip preparations, obtained from rats injected with Ca^{45} 48 hours before the experiment. This observation was reassuring, for it was demonstrated that one of the components of the cardioglobulin system contains strongly bound Ca^{++}, which is involved in the activity of the system.

Some doubts arose against the validity of this hypothesis, however, when it was found that the presence of the cardioglobulin system under natural

conditions is not restricted to the myocardium among contractile tissues, but also occurs in skeletal muscle, which allegedly is free of the staircase phenomenon. In order to resolve these contradictions, a study was undertaken to re-examine the staircase phenomenon in skeletal muscle. Ryanodine was used for the identification of the Woodworth phenomenon in skeletal muscle, after it was tested for specificity on the heart muscle of different species.

Methods: Left atrial strips of the guinea pig were 3-4 mm wide and about 1.2-1.5 mm long. The diaphragm preparation of the rat and guinea pig was dissected by Bülbring's technique with some modifications. Amphibian tissues were bathed in Ringer-Conway solution with a 3% CO_2 and 97% O_2 gas mixture. For mammalian tissues Ringer-Krebs solution was used with 5% CO_2 and 95% O_2. Both solutions were used without glucose.

Major Findings:

The Bowditch phenomenon in skeletal muscle. The stepwise development and protracted disappearance of the phenomenon in the toe muscle of the frog compared well with the one seen in many cardiac tissues. The interval-tension relation in the steady state of the same muscle demonstrates a very sharply rising contractile force from the 10-second interval towards the shorter interval range.

The presence of Bowditch phenomenon in the skeletal muscle of mammals at the change of interval between stimuli was readily recognizable.

The Woodworth phenomenon in skeletal muscle. While the development of the Woodworth phenomenon is slow in cardiac muscle, as it requires a 120-300 second rest for maximal effect, in guinea pig diaphragm the same time varies between 5-10 seconds, in the rat diaphragm between 1-2 seconds and in the toe muscle of the frog between 1-1.01 seconds or even less.

Effect of ryanodine on the Woodworth phenomenon. In order to ascertain the specificity of ryanodine, its effect on the interval-tension curve of the hearts known to have the Woodworth phenomenon was compared to those showing no sign of this phenomenon. A good example of the latter type of myocardium is the frog ventricle. It was found that the interval-tension relation remained unchanged after treatment with ryanodine. The guinea pig has the peculiar quality that in the same heart the atrial muscle has and the ventricular muscle does not have the Woodworth phenomenon. Ryanodine abolished the Woodworth phenomenon, leaving the Bowditch intact in the atrium, while on the other hand no visible effect of ryanodine was observed on the ventricular muscle. In summary, it may be said that ryanodine has a specific affinity for the system responsible for the Woodworth phenomenon, without detectable effect on any other function of cardiac tissue.

Mechanism of the Woodworth phenomenon. As is known, the cardiac muscle depends upon the extracellular Ca^{++} for its contractility on the beat-to-beat basis. On the other hand, skeletal muscle functions very competently in the absence of extracellular Ca^{++}. The rat diaphragm, stimulated once every minute,

retains good contractility even after a 7-hour perfusion in a Ca^{++}-free Krebs-Ringer solution. A faster rate of stimulation (6/min), however, reduces the resistance of the muscle towards the Ca^{++}-free perfusion considerably, since the contractility declines to 20% of the control value in 4 hours. The fastest rate of stimulation that an artificially perfused diaphragm can endure for a longer period of time is 12/min. At this rate of stimulation, the muscle loses its contractility in about 3 hours (200 ± 20 min SD obtained on 12 muscles). Thus a diaphragm with frequent stimulation, if perfused long enough with a Ca^{++}-free solution, will lose its contractility completely. Replacement of 2.5 mM/L Ca^{++} in the perfusion solution re-establishes the normal contractility in a short time. This furnishes evidence that the disappearance of contractility was due to the loss of bound Ca^{++} needed for the electromechanical coupling. A diaphragm after recalcification behaves no differently from a normal one, not even when it is exposed to a Ca^{++}-free medium.

During the process of steadily declining contractility due to Ca^{++}-free perfusion, the Woodworth phenomenon shows a similar decline of activity, as the recovery of the Woodworth phenomenon takes more and more time, as the perfusion in the Ca^{++}-free medium progresses. Under control conditions (0 hour) the Woodworth phenomenon reaches its maximum at 2 seconds. After 2-1/2, 4 and 5 hours of perfusion with Ca^{++}-free solution, this time is prolonged to 30, 60 and 300 seconds. The last stage (5 hours) exhibits a very slowly rising Woodworth phenomenon, similar in appearance to that observed in the ventricular muscle of the rat under freshly excised conditions. This demonstrates how much more strongly developed this phenomenon is in skeletal muscle than even in those cardiac tissues in which it is strongest.

By the time the Woodworth phenomenon has disappeared, all the contractility of the muscle has been eliminated with it, as if the underlying mechanism for the Woodworth phenomenon and for the bound Ca^{++} involved in the electromechanical coupling were one and the same. If these two phenomena are indeed the same, one would expect that ryanodine, which specifically and permanently destroys the Woodworth phenomenon in the heart, would also abolish the contractility of the diaphragm by interfering with the normal function of this bound Ca^{++}. This hypothesis was put to an experimental test by the following procedure.

First the rat diaphragms were decalcified in a Ca^{++}-free medium until the contractility decreased to 20% or less of that of the control level. Next the muscles were recalcified for a 20-minute period in a solution containing 0.5 mM/L Ca^{++} containing 4 μc/ml Ca^{45}, with very frequent change of Ca^{++}-free Krebs-Ringer solution for 15-30 minutes in order to eliminate the Ca^{45} not bound to the muscle. Finally the muscles were placed in successive baths containing 5 ml Ca^{++}-free Krebs-Ringer solution exchanged every 10 minutes. Ca^{++} efflux was measured by measuring the counts in the baths and expressed as % of all the counts in the muscle at the beginning of every 10-minute collection period. The Ca^{45} efflux values do not follow a straight line during the first 60 minutes of the measurements showing that at least at that time more than one component was involved in the measurements, then the efflux curve follows a straight course. If at that point 5 μg/ml ryanodine is introduced into each

765

successive sample, after a short latency there is a rapid decline of contractility, with a concomittant efflux of extra Ca^{45}, which reaches its peak at just about the time at which the tension declines to zero. The extra Ca^{45} efflux terminates with a very steep end in spite of the continuous presence of ryanodine, as if all the Ca^{45} which was ryanodine-sensitive had been mobilized.

The muscle which lost its Ca^{++} due to Ryanodine does not show any contractility even after 6 hours of perfusion with Ca^{++}-free solution free of ryanodine. Furthermore, if Ca^{++} is replaced in the bathing solution, a sudden contracture occurs, the height of which is directly proportional to the added amount of Ca^{++}. The onset of contracture is rather fast, but quickly reversible upon elimination of the Ca^{++} from the bath.

Proposed Course of Project: I propose to study the physical and chemical characteristics of the system responsible for the Woodworth phenomenon of the heart and skeletal muscle with the final aim of identifying it with the cardioglobulin system.

Honors and Awards: None

Publications: Hajdu, S., Maximin, T. J. and Leonard, E. J.: Cardioglobulin: Separation, characterization, and assay of the individual components. Circulation Research XXII: 517-526, April 1968.

Leonard, E. J., Maximin, T. J. and Hajdu, S.: Cardioglobulin: Tissue localization and plasma activity with special reference to cardiovascular disease and lupus erythematosus. Circulation Research XXII: 527-536, April 1968.

766

LABORATORY OF METABOLISM

SUMMARY
July 1, 1967 - June 30, 1968

As in past years, the Annual Report is submitted in two sections, one from the Section on Metabolism and one from the Section on Chemistry. The collaboration between the two groups has continued to be very close and very productive. The further studies on structure and metabolism of branched-chain fatty acids in relation to Refsum's disease has been a major area of interaction; a second area has been in new studies of the metabolism of alpha-tocopherol. Over the past year the newly acquired LKB-9000 combined GLC mass spectrometer has amply proved its value. Housed on the 5th floor, in the Section on Metabolism, this instrument has been a prime factor in further elucidation of the pathway for phytanic acid metabolism and in studies of alpha-tocopherol metabolism. Its availability has reduced the previously almost intolerable demands on the high-resolution MS-9 spectrometer. Moreover, the simplicity of operation of the LKB and its valuable tie to the gas-liquid chromatograph has made it even more useful than the MS-9 for some problems. The past year has also seen renewal of collaboration between the Laboratory of Metabolism and the Laboratory of Molecular Disease in kinetic studies of triglyceride metabolism in man.

The Chief of the Laboratory, Dr. Daniel Steinberg, is submitting his last annual report, having accepted a position at the School of Medicine, University of California at San Diego, beginning July 1, 1968. Because several of the research projects in progress will still be incomplete as of that date, and because several junior people working on them are committed to spend additional time at NIH, Dr. Steinberg will continue to guide these people and their projects until about January 1969. This will bring the projects to a suitable termination point and avoid wasteful disruption of research already carrying an investment. This proposal for transition has been approved by the Scientific Director, NHI, and by the Dean of the School of Medicine, UCSD. Responsibility for the Laboratory of Metabolism will be assumed July 1, 1968 by Dr. Donald S. Fredrickson, and arrangements have been made for smooth transition of responsibility.

Section on Metabolism

A. Metabolism of Phytanic Acid in Relation to Refsum's Disease

Gratifying progress has been made over the past year in this problem. (1) The pathway for degradative metabolism previously proposed has been further validated and a new step has been identified: alpha-hydroxyphytanic acid has been shown to be an intermediate between phytanic acid and pristanic acid. The alpha-hydroxylation step has been demonstrated in cell-free preparations of rat liver, the first in vitro demonstration of the alpha-hydroxylation of fatty acids and the first demonstration of the occurrence of alpha-hydroxylation of fatty acids outside the nervous system. (2) The specific site of the enzyme deletion in this recessive inherited disease has been further defined. Studies in patients and in fibroblast tissue cultures show that the enzyme error lies in an inability to carry out the alpha-

hydroxylation reaction. (3) Evidence has been obtained that the enzymatic error in all clinically diagnosed cases of Refsum's disease with phytanic acid storage is probably the same. This was accomplished by obtaining skin biopsies from bona fide cases, mostly in Europe, and initiating tissue culture. Studies in 12 such tissue cultures showed the same striking defect in oxidation of phytanic acid. (4) For the first time, it has been possible to show that heterozygotes do indeed have a partial enzyme defect. This was done using fibroblast tissue cultures derived from skin of presumed heterozygotes (biopsies shipped from Europe) and finding that the rates of phytanic acid oxidation were distinctly below those seen in normal fibroblast cultures (20-50% of normal), but not so severely depressed as in the cultures of homozygotes (less than 5% of normal). (5) Follow-up studies of patients on diets designed to reduce their stores of phytanic acid confirm that phytanic acid has an exogenous origin. The two patients studied here in the Clinical Center improved with regard to nerve conduction velocity and some tests of muscle strength and coordination. Other neurologic manifestations (hearing, vision) showed no change. The results are still inconclusive, but encourage us to pursue the problem long enough to get a definitive answer as to the therapeutic value of the special diet.

1. Basic Studied on Phytanic Acid Metabolism: The pathway previously proposed for phytanic acid breakdown in mammalian systems has been validated. Extensive studies using cell-free preparations of rat liver show that the relevant enzymes are all found in the mitochondrial fraction. Analysis of labelled products showed the formation of products previously identified in in vivo studies, and also two new degradation products: (1) alpha-hydroxyphytanic acid, and (2) the 2,3-unsaturated form of pristanic acid (pristenic acid). The yield of the latter compound was greatly increased when DPN was omitted. Pristenic acid is a predicted intermediate in the usual beta-oxidative pathway for further degradation of pristanic acid. The surprising thing is the extent to which it accumulates. In the absence of DPN, as much as 50% of added pristanic acid substrate can be converted to pristenic acid. Evidently this branched-chain acid has a low affinity for enzyme surface and is released as a free intermediate in significant amounts, unlike the intermediates in beta-oxidation of straight-chain acids, which do not accumulate.

Further evidence for the intermediate role of alpha-hydroxyphytanic acid was obtained in human fibroblast cultures and also in in vivo studies using phytanic acid-fed mice.

It was shown that alpha-hydroxyphytanic acid is metabolized in isolated mitochondria, in fibroblast tissue cultures, and in man after intravenous injection. In the mitochondrial system, the products formed from labelled alpha-hydroxyphytanic acid were the same as those previously found after incubation with labelled phytanic acid, supporting the role of the hydroxy acid as an obligatory intermediate.

On the basis of the several approaches, we can not expand the proposed pathway for phytanic acid catabolism as follows:

3,7,11,15-tetramethylhexadecanoic acid (phytanic acid)

↓

2-hydroxy-3,7,11,15-tetramethylhexadecanoic acid (α-hydroxyphytanic acid)

↓

2,6,10,14-tetramethylpentadecanoic acid (pristanic acid)

↓

2,6,10,14-tetramethyltridec-2-enoic acid (pristenic acid)

↓

4,8,12-trimethyltridecanoic acid (hexahydrohomofarnesoic acid)

↓

2,6,10-trimethylundecanoic acid

↓

4,8-trimethylnonanoic acid

Previous studies showed that phytol is readily absorbed both in animals and in man and converted to phytanic acid. Because phytol is a component of the chlorophyll molecule, it was suspected that this might be a significant dietary source of phytanic acid accumulating in patients. Studies completed over the past year show, however, that the phytol moiety of chlorophyll resists hydrolysis in the intestinal tract and is very poorly absorbed. About 95% of ingested phytol appears in the feces still linked to the porphyrin ring. Patients with Refsum's disease as well as normal subjects were studied and the degree of absorption was comparably small in both. Results were similar whether tracer doses of radioactive pheophytin a were administered or large quantities of whole cooked spinach.

During absorption of phytol a significant fraction is converted to phytanic acid. Study of the lipids in lymph showed that there was a significant amount of phytenic acid formed, but this was distributed among 5 different isomers. The chemical structure of these isomers has now been clearly established through the use of mass spectroscopy and NMR spectroscopy. Although these isomers can be formed under alkaline conditions from the major isomer (delta^{2}-phytenic acid) the methods used (acid hydrolysis) have been shown to yield only very small amounts of isomeric compounds. It is now well established that the isomers isolated in lymph are formed enzymatically. Studies in germ-free rats eliminate the possibility that intestinal flora play any major role in their formation.

2. Further Definition of the Precise Enzyme Defect in Refsum's Disease: Last year we reported studies showing that the metabolic block lay between phytanic acid and pristanic acid. The metabolism of pristanic acid was normal in patients and in their tissue cultures, whereas the metabolism of phytanic acid was markedly depressed. Having demonstrated, as just discussed, the role of alpha-hydroxyphytanic acid as an intermediate, we applied a similar approach to try to establish whether the enzyme block lay prior to formation of alpha-hydroxyphytanic acid or subsequent to its formation. Labelled alpha-hydroxyphytanic acid was synthesized in the Section on Chemistry and used as substrate for these studies. Because the chemical synthesis yields mixtures of optical isomers at the 2 and 3 positions, it was necessary to carry out several control studies which are discussed in more detail in the individual project reports. The data obtained are all compatible with the conclusion that patients with Refsum's disease and fibroblast cultures

derived from their skin oxidize alpha-hydroxyphytanic acid at a rate not significantly different from that seen in normal control subjects and their fibroblast cultures. Thus it appears that the enzyme specifically affected in Refsum's disease is involved in the introduction of the alpha-hydroxyl function into phytanic acid.

3. Generality of the Conclusion that Deletion of the Alpha-oxidative Pathway is the Common Genetic Error in Refsum's Disease: Because Refsum's disease is a rare entity, it has been difficult to carry out clinical studies in more than a few patients. One new case was studied in collaboration with Dr. Philippe Laudat in Paris. Labelled phytanic acid was shipped to Dr. Laudat who administered the material and shared with us the analysis of respiratory CO_2 and serum samples. This French patient, like the two Irish patients studied here in the Clinical Center, oxidized phytanic acid at less than 5% the normal rate. These long-distance collaborations are difficult to arrange, and so we took a different approach in order to obtain data on more cases.

Dr. Herndon, after accompanying our two Irish patients back to Belfast, visited several major European centers and obtained skin biopsies both from patients and from their close relatives. These biopsies were air-shipped to Bethesda and cell cultures initiated. In this way we were able to study in our local ward the metabolic error in 12 clinically diagnosed cases of Refsum's disease "patients in glass". All show rates of phytanic acid oxidation less than 5% of normal. The results support the tentative conclusion that all or most cases of Refsum's disease with phytanic acid storage share a common mutation.

It should be noted, however, that at least one case has been described in the literature as a case of Refsum's disease on clinical grounds even though the patient does not have phytanic acid accumulation. Our studies in fibroblast cultures from this patient's skin showed a normal phytanic acid oxidation; clinical studies done in collaboration with Dr. Herbert Kayden in New York, again showed normal phytanic acid oxidation. Another puzzling case is that of a New Zealand woman whom we have studied in collaboration with Dr. Ian Prior. She was found to have high levels of phytanic acid in her blood some years ago, but subsequent analyses showed much lower values, and then none at all. She died of unrelated causes and the tissues at post-mortem contained no phytanic acid. A skin biopsy had been obtained prior to her death, and the cultures cells oxidized phytanic acid at a rate somewhat lower than the average for normal controls, but much higher than that seen in homozygous cases of Refsum's disease. These aberrant cases do not fit into a simple classical picture for a Mendelian recessive disease. They raise the question of potent environmental influences on expression, and further studies are needed in an attempt to understand these potentially important observations.

4. Demonstration of the Carrier State in Refsum's Disease: Eight cell cultures from parents or clinically unaffected siblings of cases with phytanic acid storage were studied. In 6 of the 8, the rate of phytanic acid oxidation was less than 50% of control values. In these cases, the observed rates were below the lowest seen in control cultures i.e. there was no

overlap. The results in the other two were lower than the mean of normal values, but overlapped the lower range of normal values. These results show, for the first time, that there is a partial enzyme defect in heterozygotes.

This finding is of particular interest inasmuch as two instances have been noted in the literature in which presumed heterozygotes, clinically normal, have had some increase in plasma phytanic acid levels. It may be important to be on the alert for the possibility that some heterozygotes may not only accumulate phytanic acid but also develop clinical manifestations. The finding could also be valuable in genetic counseling. Based on these new results, attempts can be made to devise a simpler loading test for identification of the carrier state.

5. Effects of Phytol-free, Phytanic Acid-free Diet on Phytanic Acid Accumulation and Clinical Course: It is now well established that appropriate dietary restrictions can lead to reduction of body stores of phytanic acid in patients with Refsum's disease. As reported last year, there is some evidence of clinical improvement as manifested by increase in nerve conduction velocity and improvement in muscle strength and coordination. A complete battery of objective tests was applied in cooperative studies with NINDB (by Dr. W. King Engel and Dr. Frederic Q. Vroom), and it is planned to repeat this battery of tests after an additional year or so on the diet in the case of the two Irish siblings studied here last year. Dr. Laudat will study his patient in Paris in a similar manner. Because of the natural history of Refsum's disease, characterized by spontaneous relapse and remission, it is too early to conclude that the diet has therapeutic value, but the results are sufficiently encouraging to warrant continued studies.

B. Studies of the Metabolism of Myelin

Our initial hypothesis regarding the pathogenesis in Refsum's disease was based on the unique branched-chain structure of phytanic acid. It was pointed out that this molecule might not "fit" into the normal, very regular myelin structure and if incorporated could lead to the production of unstable myelin.

Studies were initiated this year to try to establish whether feeding of phytol or phytanic acid might affect the normal slow turnover of myelin. Preliminary results suggest that in fact the turnover of myelin is faster in phytol-fed rats. This approach could be quite valuable and is being pursued. Even though we have not been able to reprodce the nerve lesions of Refsum's disease experimentally by feeding phytol, it may be that a combination of phytol-loading and additional insult in the form of viral infection (which often precipitates relapses in patients with Refsum's disease) may make it possible to develop a suitable animal model.

C. Studies of Triglyceride Turnover in Plasma Lipoproteins.

Despite a great deal of study, there is still uncertainty as to the mechanism(s) underlying the various clinical forms of hyperlipidemia. Type I seems clearly to be attributable to impaired removal rates. The mechanism

771

in the other types is uncertain.

In collaboration with the Laboratory of Molecular Disease and the Mathematical Research Branch, NIAMD, comprehensive studies of triglyceride turnover are being pursued. In these studies, the kinetics of triglyceride turnover in each of the major lipoprotein fractions is being separately studied. Basic data have been defined in normal subjects, confirming that triglyceride turnover in normal individuals is relatively slow compared to the simultaneous measured turnover of FFA.

Preliminary results in patients with Type IV hyperlipemia show that the rate constant for removal of very low density lipoprotein triglycerides (VLD-TG) is much lower than normal. On the other hand, the net turnover (rate constant x pool size) is not clearly different from that in normals. This result suggests that the primary derangement cannot be overproduction, contrary to conclusions reached by others. If further studies bear out these initial findings, attention would have to be focused on mechanisms of triglyceride removal, rather than mechanisms for production and secretion of lipoproteins.

Application of this kinetic approach to the study of patients with Type III hyperlipidemia were undertaken. It was shown that the abnormal beta-lipoprotein that characterizes this type of hyperlipidemia behaves kinetically in a fashion similar to that of normal beta-lipoprotein. Elucidation of the kinetics here could give valuable insight into the underlying mechanism.

Animal studies were carried out with regard to the origin of plasma lipoprotein triglycerides. There has been controversy as to the extent to which lipoproteins produced in the liver enter the blood stream directly or indirectly by way of hepatic lymph. Rats were surgically prepared for separate collection of intestinal lymph and thoracic duct lymph. It was shown (a) that very little radioactivity from intravenously injected FFA finds its way into thoracic duct lymph, and (b) that most of this comes by way of the intestine rather than from the liver. Finally, it was shown that the extent of labelling of plasma glycerides after injection of labelled fatty acids was not significantly reduced by diversion of thoracic duct lymph. It is tentatively concluded then that lipoprotein glycerides enter the blood stream directly from the liver rather than indirectly by way of hepatic lymph.

D. Studies of Hormonal Mechanism Regulating Mobilization of Adipose Tissue Triglycerides

Previous studies from this laboratory showed that a variety of hormones stimulating fatty acid release from adipose tissue exerted their effects by increasing the activity of a hormone-sensitive lipase. A number of lines of evidence show that cyclic-3',5'-AMP mediates the action of these hormones (epinephrine, norepinephrine, ACTH, glucagon, vasopressin and others). During the past year, the critical importance of the ionic environment in regulating hormone action on adipose tissue has been further documented. A potentially important finding is that calcium ion is an obligatory requirement for the action of ACTH, but not for that of glucagon. Omission of calcium had only small effect on the lipolytic action of epinephrine. The dissociation demonstrated implies that there may be different receptors or, at the very

least, that the binding of the different hormones to receptor involves rather different associations.

Earlier studies showed, paradoxically, that cyclic AMP under some conditions inhibited lipolysis rather than stimulating it. Omission of calcium and magnesium from the medium, however, allows reproducible demonstration of a lipolytic effect of cyclic AMP. Addition of either calcium or magnesium markedly decreases the lipolytic effect of the nucleotide. Studies of the uptake of radioactive cyclic AMP in the presence and in the absence of divalent cations show that the effect of these is not through an influence on total uptake of the cyclic nucleotide.

An important advance has been made toward elucidating intimate mechanisms of hormone action by the development of a cell-free preparation of adenyl cyclase that responds dramatically to ACTH, epinephrine and glucagon. The preparation can be stored for up to 8 days without loss of activity or loss of responsiveness to hormone addition. This preparation now makes possible detailed studies of the mechanisms that underly hormone stimulation of adenyl cyclase. Similar cell-free preparations of adenyl cyclase have been prepared from liver and heart, and these preparations are likewise hormone-sensitive.

Enzyme preparations with adenyl cyclase activity appear to generate a potent inhibitor of the action of cyclic AMP. The formation of the inhibitor is enhanced under conditions that enhance formation of cyclic AMP itself. The nature and the physiologic role of the inhibitor remain to be determined, but preliminary evidence suggests that it is in fact a metabolite of cyclic AMP.

Further elucidation of the intimate mechanism of lipase activation will require purification of the enzyme. Previous attempts in this laboratory have been only partially successful. The problem has been taken up again and an approach that includes extraction of lipids from crude fractions has yielded some solubilization of lipase activity. The studies have been facilitated by development of a sensitive and reproducible micro-assay based on the use of radioactive triolein. The major problem here stems from the extremely low protein and enzyme content of adipose tissue, and successful purification will likely depend on the availability of large quantities of starting material.

E. Studies of the Metabolic Effects of Free Fatty Acids in Plasma

Last year we reported the development of a new technique for direct infusion of free fatty acids into plasma at high levels. The method rests on the use of a continuous-flow blood centrifuge developed by the National Cancer Institute in cooperation with IBM. This centrifuge continuously separates blood cells from plasma at flow rates up to 200 ml per minute. The plasma emerges from the centrifuge head in one line and the packed cells (with trapped plasma) in a separate line. Concentrated micellar solutions of salts of fatty acids can be introduced into the plasma line without harmful effects. The FFA become tightly bound to albumin before cells and plasma are recombined. Thus the formed elements are protected from the detergent properties of fatty acid anions. Solution of this technical problem makes it possible to undertake a number of research problems hitherto approach-

able only in an indirect fashion. For the first time it is possible to ask what the direct effects of FFA are in a single-variable experimental design.

One of the first problems explored was that of the interaction between FFA metabolism and glucose metabolism. The work of Randle and coworkers showed that FFA suppress glucose utilization in isolated skeletal and cardiac muscle. Based on these results, it was postulated that uncontrolled FFA mobilization might be primary in the pathogenesis of diabetes. Our initial results showed unexpectedly that dogs given continuous infusions of FFA developed hypoglycemia. Had there been an inhibition of glucose utilization, the opposite result would have been anticipated. Studies over the past year have shown that the FFA infusion leads to marked increases in blood insulin levels as measured by radioimmunoassay. Demonstration of higher insulin levels in pancreatic vein blood during FFA infusion shows that the mechanism is one of stimulation of insulin release rather than inhibition of its removal.

These new findings introduce a new metabolic control mechanism that must be evaluated before the significance of fat mobliization can be placed in proper perspective. The magnitude of the effect is related to the magnitude of FFA elevation, and studies are in progress to obtain additional data on this quantitative relationship. The physiologic significance of the phenomenon remains to be established, but it is noteworthy that insulin is a potent inhibitor of lipolysis in adipose tissue. Consequently a mechanism by which insulin is released in response to high FFA levels might operate as a classical feed-back control mechanism.

Additional research problems made possible by development of this new method include studies of effects of FFA on lipoprotein production and on ketone body production. The continuous flow centrifuge also should permit experiments in which the plasma environment is rapidly and radically altered (e.g. removal of beta-lipoproteins or albumin).

F. Further Studies of Free Fatty Acid Transport and Utilization

Comprehensive physico-chemical studies of the binding of long-chain fatty acids to albumin have been carried out. Basic data for binding have been collected and analyzed using computer techniques. These data are the first to be obtained using bovine albumin, and should be valuable to many investigators who use bovine albumin in their studies.

It has been observed that the ultraviolet fluorescence of bovine albumin is reduced when FFA become bound. This effect may be valuable in monitoring reactions involving the binding of FFA to albumin.

Further studies of the utilization of FFA by Ehrlich ascites tumor cells were carried out, showing that utilization increases as the pH of the medium is lowered from 7.4 to 6.6. Studies of FFA binding over this pH range show that the affinity of FFA for albumin decreases over this same pH range, suggesting that the increased utilization may reflect increased concentrations of FFA anions in the medium.

G. Studies on the Metabolism of Alpha-Tocopherol

Alpha-tocopherol has a side-chain closely related in structure to that of phytol. The work of Simons suggested that the degradation of this side-chain might be initiated by omega-oxidation. Were this to occur, the subsequent breakdown of the side-chain would be analogous to the breakdown of pristanic acid. This led us to explore the metabolism of radioactive Vitamin E in experimental animals.

It quickly became apparent that the Vitamin E molecule is so unstable that even the most careful handling leads to generation of acidic products. It was not possible to demonstrate with any assurance the formation of products with degraded side-chain, and these studies have been temporarily abandoned.

In the course of this work, a new non-polar intermediate was detected during thin-layer chromatography. This product was purified and carefully characterized by mass spectroscopy. It proved to be a modified dimer of alpha-tocopherol with a molecular weight of 858 (molecular weight of alpha-tocopherol equals 430). The dimerization of alpha-tocopherol under oxidative conditions has been known for some time, but there is still uncertainty as to the precise molecular structure of the product. Previous workers have also noted formation of dimer in vivo but again the exact molecular structure was uncertain. In this respect, we can be confident only that at least one product obtained after intravenous injection of 1 mg of alpha-tocopherol is a compound with a molecular weight of 858. Several additional compounds were seen on TLC, one of which is evidently a trimer also previously reported from other laboratories.

The physiologic significance of these studies remains in doubt. When tracer doses were fed to animals, it was not possible to find dimer in the liver. Bieri has suggested that the dimer formation that has been demonstrated in vivo may be artefactual and reflect only non-enzymatic conversion. Our negative results in the feeding experiments, as contrasted with the intravenous injection experiments, are consonant with this view.

Laboratory of Metabolism
Section on Chemistry

The Section on Chemistry is mainly concerned with structural analysis, synthesis and biosynthesis of compounds of biological origin. The Section also undertakes the development of new analytical techniques for compounds of biological interest.

Mass spectroscopy continues to overshadow other techniques in its application to biological problems. The earlier requirement for very pure materials has been largely obviated by the technique of combined gas chromatography-mass spectrometry using the LKB combined instrument. This instrument has been wholly satisfactory and a wide variety of problems has been studied with it, ranging from the "NIH shift" (see report of LCB) to metabolites of phytanic acid in Refsum's disease.

High-resolution mass spectroscopy continues to be a major effort in the section, particularly in collaboration with members of the computer group (CDPB-DCRT). The heuristic approach in reassembling the wealth of data from a mass spectrometer to a meaningful chemical structure or partial structure is particularly appealing. To facilitate the data handling DCRT is purchasing a small computer system (currently on bid) to be installed in our laboratory. The assembly of computer, high-speed tape punch, and plotter will be capable of processing data completely by itself, or for greater efficiency, will prepare data in a form more easily assimilated by the remote IBM 360. There is every reason to believe this same system can also be utilized to process other data from nearby instruments (NMR, ORD, IR, UV, GLC, etc.), either through tape or directly on line.

The Arthritis Institute has also recently acquired a high-resolution mass spectrometer resulting in a considerably lighter load on our high-resolution instrument. This group continues to utilize our LKB facility heavily.

The Cary Optical Rotatory Dispersion and Circular Dichroism instrument has generated considerable interest in nearby laboratories and is in full time use, frequently with a back-up schedule of one month.

The 100 Mc-Varian nmr instrument is also in constant use and has been giving satisfactory results, yielding good spectra from P^{31}, Co^{59} and C^{13} nuclei in addition to proton spectra. The increased sensitivity available has been especially useful on very small samples and the ability to do double resonance and nuclear Overhouser effect experiments has proved very important.

Alone, or in combination with other groups, members of this Section have this year:

1. Continued synthesis of compounds related to Refsum's disease, particularly labeled phytanic, pristanic and α-hydroxyphytanic acid. The

latter, a mixture of four stereoisomers has been partially resolved and varying rates of oxidation in vivo have been observed. Specifically, labeled C^1-phytanic acid has been prepared in high activity for tissue culture studies.

2. The structures of excelsine and casselsine have been elucidated and the former has been shown to be dl-cassine, implying an unusual enzymatic hydrolysis.

3. Mass spectral studies of sesquiterpenoid lactones related to Helenalin have led to useful structure spectra relationships (with L. Tsai, NHI, and W. Hertz, Florida U.).

4. The structure of peyonine (from the cactus alkaloids) has been elucidated as 3,4,5-trimethoxyphenylpyrrole-2-carboxylic acid and is apparently the first naturally occurring pyrrole alkaloid (with G. Kapadia, Howard Univ.).

5. N-ethylanhalonidine has been found to be a natural compound, one of the very few N-ethyl systems found in nature (G. Kapadia, Howard Univ.).

6. The structure of ophidine has been corrected by nmr and found to contain a more logical N-methylimidazole system rather than the reported C-methylimidazole. It is thus β-alanyl-3-methyl carnosine (J. Wolff-NIAMD).

7. The structure of N-methylcasselsine has been elucidated via a novel series of Hofmann reactions.

8. A series of programs and a system for handling mass spectra through the NHI hybrid and IBM-360 has been completed and is in partial operation (with E. Gilbert, DCRT).

9. The MS-9 mass spectrometer has been modified with regard to power supplies and amplifiers, resulting in much more reliable operation (W. Friauf, BEI-DRS).

10. A halogen-detector for gas chromatograph flame detector systems has been constructed and in preliminary tests shows surprising sensitivity (W. Friauf, BEI-DRS).

11. The LKB mass spectrometer has been interfaced with a tape recorder, making possible the eventual preparation of computer-plotted and normalized mass spectra.

12. A combined electron-impact and chemi-ionization source has been designed and contracted for, to be used with the MS-9. The simplicity of interpretation of these spectra and intensity of quasi-parent ions will be particularly useful in quantitative analysis and to determine true molecular weight.

13. A new biosynthetic pathway involving head-to-tail joining of

four carbon (butyrate units) has been discovered in Dryopteris marginalis and must be considered in other biological systems along with the established acetate-malonate pathway.

14. The mechanism of the microsomal conversion of naphthalene to its 1,2-dihydro-1,2-diol has been elucidated by using ^{18}O and mass spectroscopy. Thus the α-position is attacked first and the resulting diol is trans. Conclusions drawn from this mechanism have led to the discovery of catechol as a metabolite of 2-fluorobenzoic acid, in addition to the products previously reported (J. Holtzman, J. Gillette, LCB-NHI and P. Goldman, A&R-NIAMD).

15. The conversion of α-haloacids to their hydroxyacids in microorganisms has been found to involve H_2O rather than O_2 via O^{18} studies.

16. Various hydroxyindoles and bromoindoles have been identified from NBS cleavage of tyrosine peptides and their significance in this reaction discussed.

17. Three new nitrogenous heterocyclic systems related to alkaloids have been synthesized and their conformation determined with the aid of infrared and nmr spectroscopy.

18. The structure of a number of hydrocarbons, diterpenes and triterpene alcohols from Helichrysum dendroideum have been established and other new terpenes have been isolated from the same plant.

Serial No. NHI- 280
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project title: Study of Biological Peroxidation of Lipids

Previous Serial No.: None.

Principal Investigator: Joel Avigan, Ph.D.

Other Investigators: Leah J. Jackson

Man Years:

Total:	0.50
Professional:	0.25
Other:	0.25

Project Description:

Objectives: To study the conditions that cause lipid peroxidation in vivo; to investigate the metabolic products of unsaturated lipids formed in the course of peroxidation; to study the effect of such products on lipids and proteins.

Methods Employed: Peroxidative conditions in rats were created by administration of carbon tetrachloride, and the changes in lipids were followed by ultraviolet spectroscopy. The lipids derived from liver are to be fractionated by chromatography following chemical modification to improve the stability of the products of peroxidation.

Major Findings: In preliminary experiments a method for determination of conjugated double bonds in liver was developed. Lipids from the whole tissue or from the microsomal fraction may be used. A sample derived from a treated animal is measured spectrophotometrically against a control. The concentration of conjugated compounds in rat liver was at a maximum about 3 hours after gastric intubation and dropped to near zero at 24 hours. Fasting seemed to stimulate peroxidation. After saponification of lipids most of the conjugated products are recovered with the fatty acids. Some unknown UV absorbing material was also found, however, in the nonsaponifiable fraction. Conjugated compounds were occasionally also detected in serum lipids of treated animals, which opens a possibility for a study in humans.

Significance to Heart Research: The metabolic reactions of lipids, especially of the polyunsaturated fatty acids, has an important bearing on heart problems.

779

Proposed Course of Project: The effect of peroxidation on metabolism of polyunsaturated acids and the possible interaction of the latter with various proteins in vivo will be investigated using labeled substrates. The relationship between peroxidation and certain types of anemia in humans might also be studied, should collaboration for clinical research be available.

Publications: None.

780

Serial No. NHI- 281 (c)
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Absorption and Metabolism of Phytol and Phytanic Acid. I.

Previous Serial No.: NHI-269

Principal Investigator: James H. Baxter, M.D.

Other Investigators: Daniel Steinberg, M.D., Ph.D.
Carlos Schultz

Man Years: Total: 0.8 Patient Days--15
Professional: 0.4
Other: 0.4

Project Description:

Objectives: To study the availability of chlorophyll phytol for absorption from the intestine in normal man and in patients with Refsum's disease. (Studies in rats were described in previous report, NHI-269.)

Methods Employed: ^{14}C-Pheophytin a was isolated from tobacco leaves grown in $^{14}CO_2$ by methods previously described, and was fed to two normal subjects and to two patients with Refsum's disease. Feces were collected, and total radioactivity and phytol radioactivity of the feces were determined.

Cooked spinach (not labeled) was also fed in large amount to a normal subject, and the quantity of phytol in the feces was compared with that in the ingested spinach. In addition, cooked spinach was fed to a patient with a thoracic lymph duct fistula, and the amount of phytol (and phytanic and phytenic acids) absorbed into the lymph in 24 hours was determined.

Major Findings: (1) After ^{14}C-pheophytin had been administered, 90-95% of the administered radioactivity was recovered in the feces. 72-74% of the administered radioactivity was present in the feces still in the form of pheophytin. Only 1-2% of the fecal radioactivity migrated with free phytol.

After the fecal lipids had been saponified, radioactivity equal to 31-35% of the administered radioactivity was recovered in the nonsaponifiable fraction, and nearly all of this migrated with phytol. A comparison of the phytol recovered from the feces with that found on direct analysis of the administered material indicated that about 95% of the administered phytol had passed into the feces without being absorbed. The results were similar in normal subjects and in two siblings with Refsum's disease.

'781

(2) After the feeding of spinach (two studies), almost all of its phytol content was found in the feces. Only about 2% of the phytol (together with a somewhat smaller quantity of phytanic acid) was found in the thoracic duct lymph collected for 24 hours.

(3) It was concluded that not more than 5% of the chlorophyll phytol is absorbed by man, whether normal or afflicted with Refsum's disease. Apparently the phytyl-ester linkage in chlorophyll is resistant to the action of intestinal enzymes. Furthermore, little of the intact chlorophyll molecule is absorbed.

If a person should ingest the chlorophyll equivalent of 100 g of spinach every day for 20 years, and should absorb and retain 5% of the phytol, this could result in an accumulation of no more than 22 g of phytanic acid. On this basis, it is concluded that chlorophyll phytol is not a quantitatively important source of the phytanic acid that accumulates in Refsum's disease. (It had been suspected by a number of investigators that chlorophyll phytol might be the principal source.)

Significance to Heart Research: Free phytol is absorbed and converted to phytanic acid, which accumulates in Refsum's disease as a result of an enzyme-based deficiency in α-oxidation. Basic knowledge of the metabolism of phytanic acid may add to the understanding of cardiac physiology and pathology, since cardiac disturbances seem to occur in Refsum's disease.

Proposed Course of Project: This part of the project has been completed.

Publications:

Baxter, J. H. and Steinberg, D.: Absorption of Phytol from Dietary Chlorophyll in the Rat. J. Lipid Res., 8, 615-620, 1967.

782

Serial No. NHI-282
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Absorption and Metabolism of Phytol and Phytanic Acid. II.

Previous Serial No.: NHI-270.

Principal Investigator: James H. Baxter, M.D.

Other Investigators: G. W. A. Milne, Ph.D.
Henry M. Fales, Ph.D.
Carlos Schultz

Man Years:

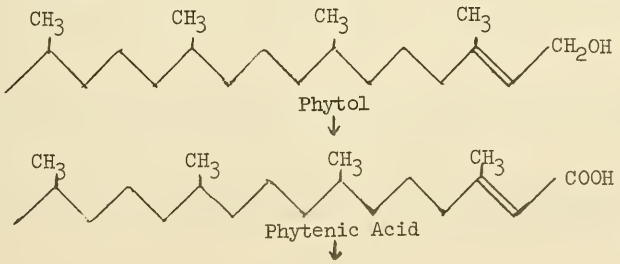
Total:	0.8
Professional:	0.4
Other:	0.4

Project Description:

Objectives: To identify the phytenic acid isomers produced from phytol by chemical means and by biological metabolism. (See previous report No.270)

Methods Employed: Methyl esters of the phytenate isomers, isolated by combined TLC and GLC from chemical preparations and from intestinal lymph of phytol-fed rats, have been examined by NMR spectroscopy and by mass spectroscopy, after oxidative (MnO_4^-) cleavage. Further studies are in progress in germ-free rats, to rule out participation of microorganisms in the formation of the isomers during absorption of phytol by the rat.

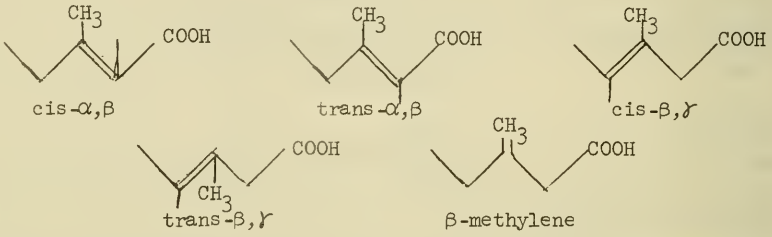
Major Findings: (1) Previous studies in this laboratory established that a major pathway for conversion of phytol to phytanic acid involves oxidation of the alcohol to a carboxylic acid as a first step, followed by reduction of the 2,3-double bond:



783

Phytanic Acid

The positions of the double bonds in the isomers found in lymph have been unequivocally established (by mass spectroscopy following oxidative cleavage), and the geometric configurations of the isomers have been determined by NMR spectroscopy. These studies identify the isomers as the cis- and trans-α,β; cis- and trans-β,γ; and β-methylene compounds:



cis-α,β trans-α,β cis-β,γ

trans-β,γ β-methylene

(2) Alkaline hydrolysis of lipids was shown also to yield 5 isomers in significant amounts, but acid hydrolysis produced only relatively little isomerization (< 5%). All evidence indicates that the isomers found in thoracic duct lymph of phytol-fed rats are enzymatically produced by the intestinal mucosal cells.

Significance to Heart Research: As stated in No. I.

Proposed Course of Project: This part of the project has been virtually completed. Phytanic acid is being isolated from intestinal lymph of conventional and germ-free rats, after phytol feeding, for determination of the diastereoisomers of phytanic acid.

Publications: None.

784

Serial No. NHI- 283
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Fatty Acid and Lipoprotein Metabolism

Previous Serial No.: None.

Principal Investigator: James H. Baxter, M.D.

Other Investigators: Carlos Schultz

Man Years:

Total:	0.4
Professional:	0.2
Other:	0.2

Project Description:

Objectives: It is known that plasma triglycerides (in the postabsorptive state) arise largely from the liver. This study is made to determine whether the triglycerides pass into the blood directly or by way of the hepatic lymph. (This is an extension of an earlier study, J. Lipid Res. 1, 158, 1965.)

Methods Employed: Since most of the liver lymph enters the cisterna chyli, it can be prevented from entering the blood by cannulating the thoracic duct beneath the diaphragm. Analyses are being made of radioactivity in thoracic duct lymph and in blood lipoproteins, after ^{14}C-labeled fatty acids have been injected intravenously in rats with thoracic duct fistulae.

Major Findings: (1) After labeled fatty acids had been injected intravenously, only about 4% of the radioactivity was found in thoracic duct lymph in 24 hours, and only about 1% in the first 4 hours.

(2) By cannulating both the thoracic duct and the intestinal lymph duct in the same animal, it was determined that most of the radioactivity in the thoracic duct lymph entered from the intestine, rather than from the liver. (However, much of it came originally from the liver, but by way of the bile into the intestine, and not by way of the liver lymph.)

(3) Initial studies indicate that the labeling of plasma glycerides, after labeled fatty acids have been injected, is not significantly reduced as a result of diversion of thoracic duct lymph.

(4) The tentative conclusion is that glycerides from the liver enter the blood directly, rather than by way of the hepatic lymph.

Significance to Heart Research: It is known that derangements in lipoprotein metabolism may cause or contribute to the development of atherosclerosis. It is necessary to understand normal lipoprotein metabolism (one facet of which is studied in this project) before the derangements can be understood.

Proposed Course of Project: Studies on labeling of plasma glycerides, as discussed above, will be completed. Efforts will also be made to rule out passage of significant fractions of hepatic lymph through channels, including the right lymph duct, that do not enter the thoracic duct beneath the diaphragm.

A related study on the dialysis of fatty acids through semipermeable membranes has been started.

Publications: None.

786

Serial No. NHI-284
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Lipolytic Enzymes of Adipose Tissue

Previous Serial No.: None

Principal Investigator: Per Belfrage, M.D.

Other Investigators: None

Man Years:

Total:	0.5
Professional:	0.5
Other:	0.0

Project Description:

Objectives: To develop methods for the purification of the lipolytic enzymes of rat adipose tissue with special attention to the hormone-sensitive lipase.

Methods Employed: New methods have been developed for the assay of lipolytic activities. These methods discriminate between lipase and esterase action and are sensitive and rapid enough to allow multiple assays during purification (e.g. fractions from a chromatographic separation). Using these assays, various procedures for obtaining triglyceride-free adipose tissue preparations have been investigated. These include ether, acetone-butanol or hexane extraction of adipose tissue homogenates or subcellular fractions taken from animals in different nutritional stares. Attempts have also been made to remove the triglycerides by allowing the endogenous lipases of the tissue to digest them in prolonged incubations. When a suitable crude lipase preparation from adipose tissue is obtained, it will be used as starting material for further purification steps such as salt fractionation, gel filtration chromatography and ion-exchange chromatography.

Major Findings: A sensitive and reproducible microassay based on the use of radioactive triolein has been developed. A new partitioning procedure considerably simplifies the assay. Pilot experiments have shown that of hexane-extracted, lyophilized adipose tissue water-homogenates may be the best starting material for preparation of the hormone-sensitive lipase. This fraction is essentially free of endogenous triglycerides and the major fraction of the protein remains soluble.

787

An esterase is readily obtained in the clear infranatant fluid after centrifugation of adipose tissue homogenates. This fraction would thus constitute a suitable starting material for purification of the esterase. However, it is clear that this enzyme, active against water-soluble substrates, is distinct from the hormone-sensitive enzyme of interest, which is a lipase, i.e. active against water-insoluble substrates.

Significance to Heart Research: Availability of plasma FFA, a chief caloric source to the heart, is regulated via the hormone-sensitive lipase activity of adipose tissue. Purification of this enzyme is essential before the intimate mechanisms of activation can be elucidated.

Proposed Course of Project: Further purification will be carried out, taking care to distinguish between lipoprotein lipase activity and hormone-sensitive lipase activity. Attempts will be made to obtain activation or inactivation of the purified preparation using appropriately fortified subcellular fractions.

Publications: None.

Serial No.-NHI-285
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through May 1, 1968

Project Title:	Myelin Metabolism in Disease
Previous Serial No.:	None
Principal Investigators:	John P. Blass, M.D., Ph.D. Daniel Steinberg, M.D., Ph.D.
Other Investigators:	Joel Avigan, Ph.D. Leah Jackson
Cooperating Units:	None
Man Years:	Total: 0.4 Professional: 0.3 Other: 0.1

Project Description:

Objectives: To compare rates of myeline synthesis in diseased animals with that in normal controls in an attempt to elucidate the metabolic responses to demyelinating agents.

Methods Employed: At appropriate intervals after the intraperitoneal injection of uniformly labelled glucose, myelin is isolated from various parts of the nervous system by density gradient centrifugation and the specific radioactivity of the myelin determined.

Major Findings: Preliminary experiments showed that the specific radioactivity of the myelin fraction at eighteen hours or more after glucose injection was similar to that in tricholoracetic acid precipitates of other brain fractions. It therefore seemed unlikely that radioactivity isolated in the purified myelin fraction was due to contamination with other tissue components.

Myelin radioactivity was found to remain more or less constant between eighteen hours and seven days in all parts of the nervous system examined (forebrain, cerebellum, spinal cord, or peripheral nerve). Specific activity appeared to be higher in spinal cord than in cerebellum or peripheral nerve, but these differences were not statistically significant.

In four animals fed a high phytol diet for two weeks, incorporation of radioactivity into myelin was significantly greater than in four controls ($P<0.001$). Animals on a high phytol diet accumulate phytanic acid in their tissues.

In four animals with experimental allergic encephalomyelitis, mean incorporation of radioactivity into myelin was higher than in four controls but the significance of its difference was marginal (P = 0.05). At this time in the course of experimental allergic encephalomyelitis, the prominent lesion is demyelination and only traces of remyelination can be detected (under the electron microscope). The results of the tracer studies suggest that the enzymes of myelination, which become repressed during the maturation of the animal, become more active again in the presence of a demyelinating stimulus.

Significance to Heart Research: Refsum's disease is associated with accumulation of phytanic acid, neurologic symptoms, and in some cases sudden death perhaps from cardiac arrhythmias; the effect of a high phytol diet on myelin metabolism may help elucidate the relationship of phytanic acid accumulation to the pathogenesis of the disease.

Proposed Course of Project: The following studies are planned or are underway: (1) effects of experimental allergic encephalomyelitis on myelin synthesis throughout the course of the disease, including recovery phases, using litter matched animals and controls; (2) effects of (asymptomatic) measles infection on myelin synthesis; (3) effect of immunizations (pertussis, vaccinia) on myelin synthesis; (4) effects of a high phytol diet on myelin synthesis, using several groups of animals under varying conditions for varying lengths of time; (5) effects of experimental allergic encephalomyelitis, infection, or vaccination on animals on a high phytol diet; (6) the relative rate of incorporation of radioactivity into various subfractions of myelin in health and disease.

Publications: None

Serial No. -NHI-286
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through May 1, 1968

Project Title:	Studies of the Hydroxylation of Straight-Chain, Long-Chain Fatty Acids in Relation to the Hydroxylation of Phytanic Acid
Previous Serial No.:	None
Principal Investigators:	John P. Blass, Ph.D., M.D. Daniel Steinberg, Ph.D., M.D. James H. Herndon, M.D.
Other Investigators:	Joel Avigan, Ph.D. Su-Chen Tsai, Ph.D. Betty Hom Ray Pittman
Cooperating Unit:	Armed Forces Institute of Pathology, Neuropathology Department
Man Years:	Total: 1.4 Professional: 1.25 Other: 0.25

Project Description:

Objectives: Previous studies in this laboratory have established that the metabolic block in patients with Refsum's disease lies in the alpha hydroxylation of phytanic acid. The important question of whether or not the pathogenesis in the disease relates to accumulation of phytanic acid *per se* remains unresolved. Another mechanism that has been considered is suggested by the high concentrations of alpha hydroxy acids as normal constituent of nerve tissue. One purpose of the present studies was to determine whether the deletion of the alpha hydroxylating system for phytanic acid metabolism was accompanied by deletion of the system for hydroxylation of straight-chain, long-chain fatty acids.

Methods Employed: Alpha hydroxy fatty acids are isolated by combinations of column chromatography (silicic acid), thin layer chromatography, and gas-liquid chromatography and by preparation of specific derivatives (copper chelates and trimethyl-silyl ethers). Quantification of the purified alpha hydroxy acids was carried out by G.L.C. of the silylated methyl esters. Concentrations of cerebrosides containing hydroxy fatty acids were determined using thin layer chromatography of total lipid extracts.

791

Studies of hydroxylation of long-chain, straight-chain fatty acids were carried out utilizing tritiated lignoceric acid (24:0). This was prepared by reduction of nervonic acid (24:1) using tritium gas. The labelled substrate was purified by thin layer and gas-liquid chromatography and the final preparation showed only a single peak of radioactivity co-chromatographing with pure lignoceric acid. The labelled phytanic acid was prepared by methods previously described (see NHI-300, 1965).

Hydroxylation of labelled lignoceric acid was studied in human fibroblast tissue cultures. The substrate was added in a mixed micellar suspension utilizing an equimolar mixture of alpha hydroxy lignoceric acid (cerebronic acid) and mono-olein.

Major Findings:

1. Concentrations of Alpha Hydroxy Acids in Tissues of Normal Controls and Patients with Refsum's Disease

Normal human skin biopsies were shown to contain the entire homologous series of alpha hydroxy fatty acids from hydroxy palmitic acid (16H:0) to hydroxyhicoso hexanoic acid (26h:0). Identification was confirmed by mass spectroscopy. Methods were developed for quantification of the very low concentrations found in the skin. The concentrations of the longer alpha hydroxy (22h:0 through 26H:0) were lower in biopsies from two patients with Refsum's disease than in four normal skin biopsies taken at operation and two autopsy samples of normal skin. While these results suggest that there may be defective formation of alpha hydroxy acids in the skin of the patients it is recognized that the unusual diet that the patients were taking (low phytol, low phytanic acid diet) may have influenced the results and a final interpretation has to be deferred.

A sample of frozen nerve obtained at autopsy from a patient with Refsum's disease and a sample of formalinized nerve from another patient were analyzed. The frozen sample was all but completely demyelinated and lipid analysis showed a virtual absence of complex lipids. The formalinized showed some decrease in connective tissue but was not grossly demyelinated. Thin layer chromatography showed a marked decrease in the cerebrosides containing hydroxy fatty acids compared either with formalinized white matter from normal nerve or with unaffected parts of the nervous system of the patient.

2. Attempts to Demonstrate Hydroxylation of Phytanic Acid in Nerve Tissue.

Tritium-labelled lignoceric acid and ^{14}C labelled phytanic acid were injected intracerebrally at the same time into weanling rats. Formation of hydroxy lignoceric acid (cerebronic acid) and of other straight-chain alpha hydroxy acids was readily demonstrated but there was no demonstrable formation of labelled alpha hydroxy phytanic acid. In another study a large dose (about 18 μc of ^{14}C labelled phytanic acid was injected into the brain of a

792

13-day old rat and again it was not possible to demonstrate formation of hydroxy phytanic acid. In this study there was radioactivity in several straight-chain hydroxy fatty acids, presumably reflecting re-cycling of radioactivity from the degraded phytanic acid into newly synthesized straight-chain fatty acids. This interpretation was supported by the findings of significant amounts of radioactivity in the animals' liver.

3. Hydroxy Acid Content of Other Tissues.

Normal human red cell ghosts were analyzed and the hydroxy acid content was found to be extremely low and variable. Because the concentrations here might well reflect differences in diet and because the concentrations were so low as to make quantitative comparisons difficult, these studies were not pursued.

Normal human sebum was also analyzed and no hydroxy fatty acids could be demonstrated.

Significance to Heart Research: Studies may lead to a definite conclusion on the essential question of pathogenesis of Refsum's disease. How the patients are to be managed would depend on answering this question and the possible relevance of the findings in Refsum's disease to other similar neurologic diseases would depend to some extent on answering this question. Among the serious manifestations of Refsum's disease are the ECG abnormalities that occur and there is evidence that some patients die suddenly, probably because of cardiac arrhythmia.

Proposed Course of the Project: Analysis of nerve tissue from patients with related peripheral neuropathies is being undertaken (Dejerine-Sottas disease); the studies of hydroxylation of straight-chain acids in tissue culture are being pursued. Further studies of noral and Refsum's tissue for hydroxy acid content will be continued to obtain more definitive results.

Publications: None

Serial No. -NHI-287
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:	Studies of Fat and Carbohydrate Metabolism during Direct Infusion of Free Fatty Acids
Previous Serial No.:	NHI-272
Principal Investigators:	Stephen R. Crespin, M.D. Daniel Steinberg, M.D., Ph.D. William B. Greenough, III, M.D.
Other Investigators:	None
Cooperating Units:	National Cancer Institute
Man Years:	Total: 2.3 Professional: 1.3 Other: 1.0

Project Description:

Objectives: To carry out direct studies of the metabolic consequences of rapid FFA mobilization taking advantage of the methods recently developed in this laboratory for direct intravenous infusion of FFA without hemolysis or other toxic side effects. To characterize FFA metabolism and the effects of FFA on lipoprotein production, ketone body production and fat mobilization. To investigate the metabolic mechanisms determining the choice of substrates (i.e. fat versus carbohydrate).

Methods Employed: The basic methods that makes possible the studies described here was developed over the past two or three years utilizing the NCI-IBM cell separator (continuous flow blood centrifuge). This device accepts blood flow rates of up to 200 ml per minute, separating the formed elements from the plasma continuously and returning them in separate lines from the centrifuge head for reintroduction into the experimental animal. Free fatty acid micellar solutions are introduced into the plasma line and given sufficient time to become complexed with albumin before cells and plasma are recombined. In this way no hemolysis is observed even in experiments extending over a period of twelve hours.

Dogs to be used in these studies are operated well in advance of their use in experiments. Indwelling A-V shunts are instituted using silastic tubing so that they may remain in place for extended periods of time. On the day of an experiment the dog requires no anesthesia, the shunt can be completely opened and connected to the continuous-flow centrifuge.

794

A radio-immuno assay for insulin has been set up based on the double antibody techniques of Morgan and Lazarow.

Major Findings: As reported last year infusions of free fatty acids caused an unexpected fall in blood glucose levels. This was the opposite of what had been expected based on the findings of Randall and co-workers showing that FFA tends to inhibit utilization of glucose by peripheral tissues. Work completed during the past year has provided an explanation for these paradoxical results. We have shown that during rapid infusion of FFA, with the accompanying hypoglycemia previously reported, there is a concomitant striking elevation of plasma insulin. Insulin levels begin to rise within fifteen minutes of the initiation of the FFA infusion and fall sharply back to control values within fifteen minutes after the infusion has been stopped. Plasma levels of over 200 micro units per milliliter have been observed. This insulin response has been seen both in fasted dogs and in dogs given continuous infusion of glucose during the course of the experiment. The magnitude of the response appears to be related to the FFA levels reached, maximum stimulation being seen when FFA levels rise to over 2 mEq per milliliter but some response being seen at lower levels. Analyses of pancreatic venous blood showed a marked increase in concentrations of insulin there and it is concluded that the response in peripheral blood insulin levels relates to an increased rate of secretion of insulin from the pancreas. Studies are in progress to determine whether the effects of FFA is mediated directly by an action on the beta cells of the pancreas or whether the mechanism is indirect.

In order to be certain that the effects seen with infusion of FFA related to what might be seen during rapid mobilization of triglycerides from adipose tissue, experiments were carried out in which glycerol was infused along with FFA. In this way we hoped to mimic the situation when triglycerides are mobilized from adipose tissue in vivo. Despite the concomitant infusion of glycerol in an amount of 1 mole for each 3 moles of fatty acid there was again a hypoglycemic response and an increase in peripheral blood insulin levels.

Sodium oleate has been used in most of the studies to date but hypoglycemia has been demonstrated also with infusions of linoleate. The present study showed that elevation of free fatty acids in the plasma introduces a hitherto undiscovered response to fat mobilization (i.e. increase in blood insulin levels). The results are not in any way in contradiction to those of Randle whose studies were largely done with in vivo systems. The in vitro studies show quite clearly that high FFA levels inhibit glucose uptake and utilization. The present studies introduce a new element, namely the increased release of insulin. Whether the net result will be an enhancement or a suppression of glucose utilization depends upon the magnitude of the two phenomena. The decrease in blood glucose levels observed in our studies could represent enhanced peripheral utilization, suppression of hepatic release of glucose or a combination of the two.

The present results are apparently incompatiblewith the conclusions reached by Schalch and Kipnis who produced an elevation of FFA levels in man

by feeding a fat meal and then injecting heparin intravenously. They observed that the rate of glucose utilization, measured by the rate constant for disappearance of an intravenous load, was decreased when FFA levels were high. We have carried out a few studies in dogs employing a protocol similar to that used by Schalch and Kipnis (i.e. feeding a fat meal to dogs who were already receiving large doses of heparin as they must while on the continuous-flow centrifuge). In these studies we did not see the striking hypoglycemic response seen with infusions of FFA even though FFA levels in some cases arose nearly as high as they did when FFA were infused directly. This difference in results is intriguing and suggests that some aspects of chylomicron metabolism introduced still another variable with regard to control of glucose utilization. Very recently Ontko and Randle have shown that the glucose utilization by a perfused rat heart is suppressed when chylomicrons are included in the perfusing medium.

Significance to Heart Research: The discovery of this new potential mechanism for metabolic control requires reexamination of some of the previously postulated mechanisms regulating fat and carbohydrate utilization. Evaluation of the significance of these mechanisms in physiologic and pathologic states remains to be determined. Because of the probable role of fat mobilization in contributing to the disordered metabolism of diabetes and atherosclerosis it would be valuable to understand more completely the place of this mechanism in metabolic rate relations.

Proposed Course of the Project: Experiments will be carried out to study further: (1) The fate of infused fatty acids; (2) the effects of moderate and short-term elevations of FFA on glucose metabolism and insulin secretion, peripheral and pancreatic; (3) the effect of sustained elevations of FFA on metabolic rate and plasma lipoproteins in dogs; (4) the effect of high FFA levels on insulin secretion and glucose metabolism of isolated pancreatic tissue; (5) the effect of other drugs and hormones on the stimulation of insulin secretion caused by high FFA levels; (6) the effect of high FFA levels on individual organs and tissues.

Publications:

Greenough, W. B. III, Crespin, S. R. and Steinberg, D.: Hypoglycemia and Hyperinsulinemia in Response to Raised Free Fatty Acid Levels. Lancet, 1334-1336, December 23, 1967.

Judson, G., Jones, A., Kellogg, R., Buckner, D., Eisel, R., Perry, S. and Greenough, W. B. III: Closed Continuous-Flow Centrifuge. Nature, 217: 816-818, 1968.

796

Serial No.-NHI-288(c)
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:	Lipoprotein Kinetics in Hyperlipemic Patients
Previous Serial No.:	NHI-271
Principal Investigator:	Arthur Frank, M.D.
Other Investigators:	Steven Quarfordt, M.D. Daniel Steinberg, M.D., Ph.D. Mones Berman, M.D.
Cooperating Units:	Laboratory of Molecular Diseases, NHI Mathematical Research Branch, NIAMD
Man Years:	Total: 2.5 **Patient Days--40** Professional: 2.5 Other: 0

Project Description:

Objectives: The project was designed to investigate the metabolism and kinetics of FFA and of the triglycerides of each of the three major lipoprotein classes in man. By using this model to compare the kinetic parameters in normals and in various hyperlipemic states, it is hoped that a better understanding of the mechanisms of synthesis and removal of triglycerides can be obtained and the type of derangement in various forms of hyperlipidemia elucidated.

Methods Employed: Studies were performed on patients with hyperlipemia and on normal volunteers. Small amounts of C^{14}-fatty acid-albumin complex were injected intravenously and a series of venous blood samples obtained at intervals thereafter. The disappearance of radioactive fatty acids from the plasma and the appearance and subsequent removal of this label in plasma triglycerides of several lipoprotein fractions was determined as a function of time.

The data were analyzed by computer techniques using the SAAM program of Berman, utilizing in this past year, the computer facilities of the National Bureau of Standards.

Major Findings: Fifteen experimental studies have now been completed in two normal subjects, three subjects with Type IV hyperlipidemia, three subjects with Type III hyperlipidemia and two subjects with Type I

797

hyperlipidemia. Construction of kinetic models based on computer analyses of the data are continuing and the following summary is based on the analysis to date.

The best fit of the data for free fatty acids (FFA) kinetics is obtained only by postulating two separate pools with which the plasma FFA pool equilibrates. The FFA are removed from the plasma via oxidation or by uptake in the liver and conversion of FFA to triglycerides.

Data for the metabolism of the triglycerides in the very low density lipoproteins (VLD-TG) have been most extensively analyzed. The multicompartmental model currently in use and fitting the data best is one in which the VLD-TG are derived exclusively from a hepatic source. The model requires however that the hepatic precursors of plasma VLD-TG are in part derived from non plasma FFA precursors (i.e. carbohydrate precursors). The extent of this contribution varies with the conditions of the study. It should be noted that one of the advantages of this type of kinetic approach is that it permits validation of the mathematical model by testing for compatibility with the observed tracer concentrations as well as with the steady-state concentration of the relevant factions. The formulation of the final model and proposals such as outlined above require consistency with both types of data.

A few studies have been carried out comparing the metabolism of lipoprotein triglyceride in the same subject when on a regular diet and when on a high carbohydrate diet. Study of these results is currently underway and the data are too limited to permit final conclusions. Preliminary analysis, however, suggests that in normal subjects there is an increased production of triglyceride when on a high carbohydrate diet but that this is accompanied by an increased rate of removal with the result that plasma levels do not rise very significantly. Several studies have been carried out in patients with Type III hyperlipidemia. These patients, as shown by Levy, Lees and Fredrickson, have a significant amount of lipoprotein with density less than 1.006 (i.e. like that of the VLD) but having the characteristics of beta lipoprotein. The tracer kinetics of the "floating beta" fractions appear to be very similar to those for the ordinary beta lipoprotein. Further studies of these interesting cases may help elucidate the biochemical origin of this peculiar "floating beta" fraction.

Significance to Heart Research: Hypertriglyceridemia is associated with increased incidence of coronary artery disease. The underlying mechanism of hypertriglyceridemia is still uncertain except in the case of patients with Type I hyperlipemia. These studies if they lead to elucidation of the mechanism might provide a basis for more rational therapy for hyperlipidemia states and thus to rational therapy of a prophylactic type for patients with arteriosclerotic heart disease. The rate constants for removal of VLD-TG from the plasma compartments ranged from about 0.01 to 0.02 in normal subjects. In patients with Type IV hyperlipidemia the rate constant for removal was considerably lower. On the other hand the pool size for VLD-TG in these patients is much larger than normal. The calculated net turnover of TG is in the patients not clearly different from that in normals. These results do not support previously calculated mechanisms involving over-production of

VLD-TG as the primary mechanism in Type IV disease; they are compatible with an abnormality in removal of VLD-TG from the plasma compartment.

Of great interest is the kinetic relationship between VLD-TG and LD-TG (beta$_1$ lipoprotein triglyceride). The data are compatible with a strict precursor-product relationship between the triglyceride in these two factions. A similar precursor-product relationship is suggested between VLD-TG and HD-TG (high density, alpha, lipoprotein triglyceride).

Project Course of Project: The various models will be further evaluated and data will be obtained to clarify and reconcile the precise nature of each of the measured components. As each segment of the model is completed, it will be merged with other components to construct a complete model of fatty acid-lipoprotein interrelationships.

Publications: None

799

Serial No. NHI-289
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:	Refsum's Disease: Further Studies in Tissue Culture.
Previous Serial No.:	NHI-273
Principal Investigators:	James H. Herndon, Jr., M.D. Daniel Steinberg, M.D., Ph.D.
Other Investigators:	B. William Uhlendorf, Ph.D. G. W. A. Milne, Ph.D. Henry M. Fales, Ph.D.
Cooperating Units:	Laboratory of Viral Immunology, DBS Section on Chemistry, Lab of Metabolism, NHI
Man Years:	Total 1.0 Professional: 1.0 Other: 0.0

Project Description:

Objectives: Previous studies in this laboratory had established that tissue cultures derived from patients with Refsum's disease retained the defect in degradation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) characteristic of the whole patient. Extensions of this work aimed at (1) deciding among several hypotheses regarding the precise locus of the enzyme defect, (2) confirming the generality of these findings through studies in additional patients with Refsum's disease, and (3) attempting to characterize and identify the asymptomatic heterozygotes biochemically.

Methods Employed: U-^{14}C-alpha hydroxyphytanic acid was synthesized from phytol by way of phytanic acid. The labeled phytanic acid was brominated and then hydrolyzed to alpha hydroxyphytanic acid. The product was pure by gas-liquid chromatography but was resolved into two major bands on thin-layer chromatography. The radioactivity was equally divided between the two bands. In the formation of phytanic acid from phytol, two isomers are generated at the asymmetric number 3 carbon formed; in conversion of this 3D, 3L mixture to the alpha hydroxy acid again, two isomers are formed at the now asymmetric number 2 carbon. Thus a mixture of isomeric compounds is expected and the two bands isolated by TLC are presumed each to contain two different isomeric compounds. Through the generous cooperation of Dr. Morris Kates, we obtained a small sample of radioactive 3D, 7D, 11D phytanic acid and converted this to alpha hydroxyphytanic acid. The product resolved into two TLC bands co-

800

chromatographing with those derived from the chemical synthesis described above. This result, together with the very minimal optical rotation of our synthetic alpha hydroxyphytanic acid mixtures, led to the tentative conclusion that one band contained 2L, 3L alpha hydroxyphytanic acid plus 2D, 3D alpha hydroxyphytanic acid; the other band was presumed to contain a mixture of 2D, 3L and 2L, 3D alpha hydroxyphytanic acid. The rates of metabolism of these isomeric mixtures were separately studied.

Because Refsum's disease is rare and it is impossible to carry out clinical studies in any large number of patients, scattered as they are around the world, we decided to attempt collecting and perpetuating tissue culture lines from as many patients as possible and also from presumed heterozygote relatives. Dr. Herndon, after accompanying our two Irish patients back to their home near Belfast, made visits to Oslo, Bergen, Paris and Lille. At each of these places, through the cooperation of local physicians previously contacted, Dr. Herndon obtained skin biopsies which were shipped back to Bethesda where Dr. Uhlendorf initiated the culture from them. All 19 biopsies sent were successfully started and are now growing or are available for reinitiation in Dr. Uhlendorf's tissue culture laboratory.

In the present series of studies, tritium-labeled palmitic acid was incubated in each culture bottle along with ^{14}C-labeled branched-chain acid being studied. Results for the branched-chain acid were related to the simultaneous oxidation of palmitate (measurement of T_2O released to water) thus correcting for the metabolizing mass of tissue and, hopefully, taking into account any variations in metabolic activity of the various bottles of cells.

The amount of radioactive fatty acid remaining unmetabolized in the cells at the end of the incubation was determined, and in some cases gas-liquid chromatography was used to subfractionate and determine the nature of fatty acids incorporating radioactivity.

Major Findings:

1. Further Localization of the Enzyme Defect: In last year's report, we described studies that localized the enzyme defect to one of the steps between phytanic acid and pristanic acid, the (n-1) products of alpha oxidation of phytanic acid. During the past year studies in several different systems have shown that alpha hydroxyphytanic acid is an intermediate between phytanic acid and pristanic acid. Studies of the radioactivity in fibroblasts after incubation with phytanic acid have demonstrated the presence of alpha hydroxyphytanic acid in these human cells. Formation of the alpha hydroxy acid has also been demonstrated in rat liver mitochondria and in mouse liver in vivo.

Control cultures of human fibroblasts oxidized ^{14}C-alpha hydroxyphytanic acid to $^{14}CO_2$. The rate of oxidation, expressed as percentage of substrate added to the medium, was considerably slower than that for phytanic acid. The reason for this poorer rate of oxidation is not established with certainty but there is some reason to believe that it is related to poor penetration of the hydroxy acid into the cells.

807

Recent results using rat liver mitochondria show that carnitine very markedly accelerates the rate of oxidation of alpha hydroxyphytanic acid. Direct comparison of the rate of oxidation of alpha hydroxy phytanic acid in control cultures and in cultures of cells from patients with Refsum's disease showed no significant difference even though these same cells showed a very striking disparity in rate of oxidation of phytanic acid itself. Thus, there does not appear to be any block in the oxidation of alpha hydroxyphytanic acid and it is inferred that the enzyme deleted is one involved in alpha hydroxylation. This conclusion is supported further by clinical studies reported this year in "Study of the Biochemical Defect in Refsum's Disease".

The mixture of isomers in the upper TLC bands was oxidized about 50% more rapidly than the mixture in the lower TLC bands. Similar relative rates of oxidation were found in isolated rat liver mitochondria and also in patients in vivo. When the single isomers derived from the labeled D,D,D-phytanic acid were similarly compared in the mitochondrial system, again the faster moving band was oxidized more rapidly than the slower moving band, and the difference was again about 50%. Thus, it appears that both the D and the L-alpha hydroxyphytanic acids are metabolized but that there is some selectivity in the systems involved based on optical isomerism.

2. Generality of the Enzyme Defect in Different Patients with Refsum's Disease: With the additional cell lines obtained as a result of Dr. Herndon's European trip, we now have a total of 12 patients represented in our "Refsum's Disease Ward" in the tissue culture laboratory. The rate of oxidation of labeled phytanic acid in each case is less than 5% of the rate in control cultures. All of these patients are patients that have significant accumulations of phytanic acid in their plasma. Thus it appears that all of the cases of phytanic acid storage disease thus far studied have the same mutation (i.e. lack of an enzyme system involved in the alpha oxidation of phytanic acid).

Several additional control studies have been done in order to be certain that the defective rate of oxidation of phytanic acid reflects an enzyme deletion of a direct sort. The possibilities that the defects might lie in an inability to take up phytanic acid is ruled out by the finding that cells from patients with Refsum's disease take up an amount that is equal to that taken up by control cultures. Because the patients' cells oxidize less, they actually have a higher residual content of labeled phytanic acid at the end of the incubation. A second possibility considered was that the defect might lie in an inability to release phytanic acid from ester linkage once formed. Cells were labeled with phytanic acid and then transferred to unlabeled medium. The rate of release of radioactivity to the medium was determined and found to be comparable in control cells and in cells from patients with Refsum's disease. A third possibility considered was that phytanic acid might be "toxic" to cells from patients with Refsum's disease and not to cells from other patients or normal subjects. Cells were exposed for 48 hours to graded concentrations of phytanic acid and no difference was found in the sensitivity of the cells whether they were derived from controls or from patients with Refsum's disease. These tests for acute toxicity are

802

being supplemented by studies of longer term toxicity which have the additional aim of detecting abnormal phytanic acid storage in cells from affected individuals.

3. Detection of the Carrier State: The parents of patients with Refsum's disease have never been found to have clinical manifestations nor, with two exceptions, have they been found to accumulate phytanic acid in blood. In neither case have there been clinical symptoms, but interesting questions are raised by these two examples of clinically normal parents with some phytanic acid demonstrable in plasma lipids. Some attempts have been made to identify heterozygotes by feeding large doses of phytol (1 gm), but these have yielded negative results.

Eight of the presumed heterozygote cell lines obtained by Dr. Herndon have now been studied with respect to rate of phytanic acid oxidation. Six of the eight showed clearly abnormal rates of oxidation -- 20 to 50% of the mean values in control cell cultures, values below the lowest found in control cultures. The rates in the other two cultures were well below the mean value for control cultures but overlapped the lower range of values found in controls. The results strongly suggest that heterozygotes do have a partial enzyme defect. This represents the first demonstration of a carrier state in this disease.

Significance to Heart Research: These studies strongly suggest that all patients with phytanic acid storage disease (Refsum's disease) have the same basic mutation, one affecting the system for alpha oxidation of phytanic acid. The results will be of great value in deciding on management of these patients should the dietary treatment now under investigation prove to be of value (i.e. one can proceed on the assumption that the diet being studied should be equally effective in all clinical cases of phytanic acid storage disease).

For the first time it was demonstrated that there is a carrier state in Refsum's disease; this should be helpful in the future when it is important to ascertain whether a given individual is not a carrier with respect to genetic counseling. The further elucidation of the specific enzyme error in the disease may prove valuable in further studies of its pathogenesis. Among the clinical manifestations of the disease are abnormalities in cardiac conduction leading in some to sudden death presumed to be due to cardiac arrhythmias.

Proposed Course of Project: The larger numbers of cell lines now available will be studied with regard to their capacity to oxidize alpha hydroxyphytanic acid and pristanic acid in order to ascertain whether or not the defect lies in alpha hydroxylation in all of them. Further studies will be done with the cell lines derived from heterozygotes in order to be certain whether or not the carrier state can always be detected. Attempts will be made to devise an in vivo tolerance test since the cell culture approach is time-consuming and not generally available.

803

The question of whether or not phytanic acid itself is of pathogenetic significance will be further explored. Studies are currently under way to determine whether prolonged exposure of the cells to phytanic acid leads to long-term toxicity in cells from the patients because they cannot metabolize or get rid of phytanic acid. Studies are continuing in collaboration with Dr. Donald Silberberg at the University of Pennsylvania on possible toxic effects of phytanic acid on the process of myelination. Studies will be carried out to determine whether alpha oxidation of other substrates than phytanic acid is blocked in patients with Refsum's disease. Because of the almost complete deletion of the alpha oxidation system for phytanic acid, it becomes a rather simple matter to decide whether oxidation of other substrates is carried out to a large extent by the same system that oxidizes phytanic acid using the cell culture approach.

Publications:

Tsai, Su-Chen, Herndon, J.H., Jr., Uhlendorf, B.W., Fales, H.M., and Mize, C.E. The Formation of Alpha-Hydroxy Phytanic Acid from Phytanic Acid in Mammalian Tissues. Biochem. Biophys. Res. Comm., 28: 571-577, 1967.

Serial No.-NHI-290
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:	Agents that Influence the Formation and Action of Cyclic 3', 5'-AMP in Adipose Tissue, Heart and Liver.
Previous Serial No.:	None
Principal Investigators:	Ferid Murad, M.D., Ph.D. Martha Vaughan, M.D.
Other Investigators:	Sally Stanley Ferol Lieberman
Cooperating Units:	None
Man Years:	Total: 0.7 Professional: 0.6 Other: 0.1

Project Description:

Objectives: To study the role of cyclic 3', 5'-AMP as the mediator in hormonal control of various tissues.

Methods Employed: Particulate preparations from adipose tissue, liver and heart have been used as sources of adenyl cyclase, which catalyzes the formation of cyclic 3', 5'-AMP from ATP. Cyclic 3, 5-AMP is assayed by its ability to activate liver and heart phosphorylase kinase and glycogen phosphorylase. Cyclic 3, 5-AMP and other nucleotides from cell-free incubations and tissue extracts have been purified and identified using column, paper, and thin-layer chromatography and electrophoresis.

Major Findings: A variety of hormones have been found to stimulate the formation of cyclic 3, 5-AMP in cell-free systems of heart, liver and adipose tissue. In such systems a potent inhibitor of cyclic 3, 5-AMP can be found and characterized. Studies to date indicate that this inhibiting material is a metabolite of cyclic 3, 5-AMP with similar characteristics with various techniques for purification. The nature and physiologic role of this inhibitor will be investigated.

Significance to Heart Research: Such studies offer an explanation for the mechanism of action of a variety of hormones and adrenergic blocking agents.

805

Proposed Course of Project: The identification of the inhibiting material and its mechanism of action will be pursued.

Publications: None

Serial No. NHI-291
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:	Free Fatty Acid Transport Utilization
Previous Serial No.:	NHI-276
Principal Investigator:	Arthur A. Spector, M.D.
Other Investigators:	Kathryn M. John John E. Fletcher
Cooperating Units:	Laboratory of Applied Studies, DCRT Laboratory of Physical Biology, NIAMD Laboratory of Technical Development, NHI
Man Years:	Total: 2.2 Professional: 1.2 Other: 1.0

Project Description:

Objectives: To determine the detailed mechanism by which free fatty acid (FFA) is transported in the blood and across cell membranes and is utilized.

Methods Employed: Physico-chemical techniques were employed to study the interaction of FFA with proteins and the properties of fatty acids in aqueous solutions. Ehrlich ascites tumor cells were used as a model system in order to study FFA transport and utilization in a mammalian cell preparation.

Major Findings: Association constants were derived for the binding of long-chain fatty acids to bovine albumin. The influence on binding of the pH, salt composition, ionic strength and temperature of the incubation medium has been determined. This information will aid in making detailed interpretations of *in vitro* experiments in which FFA-albumin solutions are used. In order to obtain a more precise analysis of the binding results, a computerized mathematical method for fitting the experimental data to a theoretical model was devised. This method has been applied so far only to the FFA-albumin results, but it is generally applicable for analyses of the binding of ligands to macromolecules.

The intensity of the ultraviolet fluorescence emission of bovine albumin is reduced by as much as 45% when FFA is added to the protein, and the maximum emission is shifted to a shorter wave length. Both the magnitude of quench-

807

ing and the extent of the blue shift increase as the molar ratio of FFA to albumin increases until approximately 4 μEq of FFA are bound per μmole of protein. Although many experimental problems remain to be solved, these findings at least suggest that spectrophotofluorometric methods may be useful for monitoring reactions involving FFA and bovine albumin.

The distribution (at equilibrium) of radioactive long-chain FFA between an n-heptane and an aqueous phase was measured. These data can be explained adequately without assuming association between FFA molecules in the aqueous solution.

Uptake and utilization of exogenous radioactive FFA by Ehrlich ascites tumor cells increased when the pH of incubation media containing albumin was lowered from 7.4 to 6.6. In contrast, the utilization of radioactive FFA already contained in the cells changed very little as the pH of the incubation medium was lowered over this range. In separate experiments, it was observed that the affinity of albumin for long-chain FFA became weaker as the incubation medium pH was decreased below 7.4. Thus, pH changes probably influence FFA utilization indirectly by altering the affinity of albumin for FFA. Less endogenous lipid radioactivity was utilized during incubation of cell labelled in vivo with palmitate-1-^{14}C at pH 6.8 than at pH 7.4. Hence, it appears that incubation in slightly acid media will result in a relative net accumulation of lipid in the cell. These findings suggest that extracellular pH may be one factor that is involved in the physiological regulation of FFA utilization.

Significance to Heart Research: Abnormalities in lipid transport almost certainly are involved in the etiology of atherosclerosis and, hence, myocardial infarction. Fundamental studies concerning the effects and utilization of fatty acids in isolated in vitro systems will permit a more thorough understanding of the role that lipids play in the development of these diseases.

Proposed Course of Project: This project will be terminated between August 1 and September 1, 1968.

Publications:

Spector, A. A. and John, K. M.: Effect of Free Fatty Acid on the Fluorescence of Bovine Serum Albumin. Arch. Biochem. Biophys., in press.

Spector, A. A., John, K. M. and Fletcher, J. E.: Binding of Free Fatty Acid to Bovine Serum Albumin. J. Lipid Research, in press.

Serial No.-NHI-292 (c)
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:	Study of the Biochemical Defect in Refsum's Disease (Phytanic Acid Storage Disease)
Previous Serial No.:	NHI-275C
Principal Investigators:	Daniel Steinberg, M.D., Ph.D. James H. Herndon, Jr., M.D. Charles E. Mize, M.D. Henry M. Fales, Ph.D. G. W. A. Milne, Ph.D.
Other Investigators:	Ray Pittman Betty Hom W. King Engel, M.D. Frederick Q. Vroom, M.D. Philippe Laudat, M.D. J. H. D. Millar, M.D.
Cooperating Units:	National Institute of Neurological Diseases and Blindness Laboratoire de Pathologie Medicale, Paris Claremont Street Hospital, Belfast, N. Ireland
Man Years:	Total: 2.6 Patient Days--270 Professional: 1.5 Other: 1.1

Project Description:

Objectives: Last year we reported studies localizing the enzyme defect in Refsum's disease to a system for the conversion of phytanic acid to pristanic acid. One purpose of studies done over the past year was to attempt to further define the precise nature of the enzyme defect.

Last year we reported that appropriate dietary modification would result in a decrease in the blood levels of phtanic acid in patients. Studies continued over the past year have extended these findings and given more data with regard to possible effects on clinical status.

Finally, a number of studies of the distribution of phytanic acid in other body fluids and among the various lipid classes in them have been carried out.

809

Methods Employed: Most of the methods are as described in the previous report (NHI-275C). The preparation of alpha hydroxy phytanic acid U ^{14}C is described in report NHI-294. Patient material: Two patients with Refsum's disease studied in the Clinical Center and one patient with Refsum's disease studied in collaboration with Dr. Philippe Laudat in paris. Eight normal control subject studied in the Clinical Center.

Major Findings: Additional studies done here in the Clinical Center and collaboratively with Dr. Philippe Laudat on a patient in Paris confirm the previous finding that phytanic acid oxidation is markedly depressed in patients with Refsum's disease. The rate of conversion to $^{14}CO_2$ was less than 5% of that in control subjects.

Studies of the oxidation of alpha hydroxyphytanic acid, however, showed no significant difference between control subjects and patients with Refsum's disease. These results, paralleling those obtained in tissue culture studies (see NHI-273), support the conclusion that the enzyme defect lies specifically in the alpha hydroxylation of phytanic acid. As reported previously the imposition of a diet low in phytol and in phytanic acid leads to a progressive fall in plasma levels of phytanic acid in the patient. Green vegetables were deleted from the diet because of the phytol content of chlorophyl. Free phytol has been shown by us previously to be very well absorbed by man and converted readily to phytanic acid. Recent studies by Dr. Baxter, however (see NHI-281), showed that the phytol moiety of chlorophyll is not split from its ester linkage to any significant extent during passage through the gut. At least 90 to 95% of the chlorophyl phytol remains complexed in the chlorophyll molecule and appears in the feces in that form. Consequently the necessity for eliminating green vegetables can be questioned. On the other hand there are other branched-chain compounds in vegetable products and since these may also be precursors of phytanic acid we have continued the patients on a diet free of green vegetables. One of the two patients studied on this diet continued to show a progressive fall in phytanic levels, reaching values about 1/3 the values at admission; the other subject, despite continuation of the diet, showed a rebound in phytanic levels for reasons undetermined and plateaued at a value of about 50% of the admission values. At the time the patients were returned to Ireland (October, 1967) they maintained the moderate improvement discussed in last year's report. Serum samples continue to be sent to us from Ireland for analysis and the patients are supposed to be continuing a diet like that maintained here in the Clinical Center. One of the two (J.S.) continues to maintain his phytanic levels at about the values he showed when he left the Clinical Center. His clinical status remains more or less stable. The second patient (K.S.) has shown a marked rise in her plasma phytanic levels since returning home. Dr. J. H. D. Millar, who has the patients under his care in Ireland, is unaware of any major changes in the patients diet. Nevertheless her phytanic levels have risen to the values about the same as those she showed when she first came here from Ireland. Over this past four or five months the patient has evidently deteriorated clinicall as well. For this reason, and to avoid any ambiguity in this long-term follow-up study, we have proposed to bring these two patients, in whom so much time has been invested, back to the Clinical Center so that the diet can be unquestionably controlled and reevaluate their clinical status.

8/0

Cerebrospinal fluid from one of the patients was analyzed and shown to contain significant concentrations of phytanic acid. Rectal biopsies and small intestinal biopsies were analyzed and again phytanic acid storage was demonstrated.

Studies of the distribution of phytanic acid among the various lipid classes of the plasma were carried out. Phytanic acid was present predominantly in the triglyceride and phospholipid fractions; only a very small percentage of the fatty acids in cholesterol esters were phytanic acid molecules. In none of the samples was it possible to demonstrate alpha hydroxyphytanic acid, pristanic acid, or any of the lower degradation products on the pathway for phytanic acid degradation. These negative results are consonant with the conclusion that these patients have little or no alpha oxidative pathway for phytanic acid breakdown. If there were any impairment in the activity of enzymes lower in the pathway one might anticipate seeing some accumulation of products below phytanic acid but none has been demonstrated in these studies or in any published studies of which we are aware. Dr. Engel and his collaborators have carried electron microscopic study of a nerve biopsy from one of the patients with Refsum's disease. The results establish clearly that the cellular elements comprising the so-called "onion bulb" in the peripheral nerves in this disease arise from Schwaann cells. These cells showed a striking number of osmiophilic inclusion bodies. These were obviously "crystalline" in nature (i.e. they demonstrated remarkable regularity of spacing of electron-dense particles, the spacing being approximately 40 Angstroms).

Significance to Heart Research: The further studies reported here pinpoint the enzyme defect in this new lipid storage disease. Studies are in progress to determine whether or not other pathways involving alpha hydroxylation are also blocked (see NHI-286). Whether or not phytanic acid storage is in itself sufficient to explain the pathogenesis is still uncertain. More patients need to be studied on the special phytol-free phytanic acid-free diet for a longer period of time. If the pathogenesis could be clarified the results might be relevant to our understanding of this neurologic disease and perhaps related neurologic diseases. Specifically the involvement of the cardiac conduction system which is believed to account for some of the unexplained sudden deaths in this disease it deserves further investigation to determine the underlying mechanism.

Proposed Course of Research: The clinical studies on the effects of diet on the course of the disease will be continued. Attempts are being made to interest other investigators in extending this kind of study. Dr. Philippe Laudat in Paris plans to study two new cases under his care in this way and studies are continuing in Norway along these lines. Based on the tissue culture results (see NHI-273) attempts will be made to devise a simpler test for identifying the heterozygotes (i.e. carriers of the single recessive gene). Renewed study of the possibility of producing an animal model is justified. In particular the possible exacerbating influence of viral disease will be investigated in experimental animals. Finally, it is proposed to examine the possible influence of the large concentrations of the unusual branch-chain fatty acids on lipoprotein production and lipoprotein metabolism both in patients and in experimental animals.

Publications:

Steinberg, D., Herndon, J. H., Jr., Uhlendorf, B. W., Mize, C. E., Avigan, J. and Milne, G. W. A.: Refsum's Disease: Nature of the Enzyme Defect. Science, 156: 1740-1742, 1967.

812

Serial No.-NHI-293
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:	Metabolism of α-Tocopherol
Previous Serial No.:	None
Principal Investigators:	Barry S. Strauch, M.D. Joel Avigan, Ph.D.
Other Investigators:	Henry M. Fales, Ph.D.
Cooperating Units:	None
Man Years:	Total: 1.2 Professional: 1.1 Other: 0.1

Project Description:

Objectives: Recent studies in this laboratory have revealed a block in the alpha hydroxylation step in the initiation of alpha oxidation of phytanic acid in patients with Refsum's syndrome. A pathway in these patients to oxidize phytanic acid would depend, then, on omega oxidation. The present studies were initially designed to explore such a pathway in mammals. Vitamin E (alpha tocopherol) has a branched hydrocarbon side chain (phytyl) and was used as a model to study omega oxidation. This seemed appropriate in that products of side chain oxidation of Vitamin E (acidic metabolites) are known to occur in the urine. Such acidic metabolites could only be formed by omega oxidation.

Methods Employed: Doses of radioactive tocopherol were administered either intravenously or orally to rats and mice. At appropriate times liver lipids were isolated. In early experiments these liver lipids were extracted with strong base (saponified) to search for acidic metabolic products of the alpha tocopherol. In other experiments extraction was performed with alkaline ethanol. Radioactive fractions in the liver were also isolated and purified by thin layer and column chromatography. Comparison of such isolated products was made with compounds synthesized by chemical oxidation of alpha tocopherol. Such chemically synthesized products were extensively studied by mass spectrometry, gas-liquid chromatography, and various physical and chemical methods.

Major Findings: The initial studies with saponification of the total liver lipids seemed to reveal a significant amount of radioactive acidic product. However, appropriate control experiments revealed that such products

813

were derived from artefacts of isolation with strong base. Extraction with basic ethanol failed to reveal significant formation of acidic metabolites when proper controls were employed.

Thin layer chromatography (T.L.C.) of the total liver lipids in intravenous experiments revealed a significant amount of material in a fraction less polar than triglycerides and tocopherol itself. This fraction had no correlate in controls and thus was further investigated. The fraction was further purified by T.L.C.. Direct mass spectrometry of the purified radioactive subfraction revealed a molecular weight of 858 (molecular weight of alpha tocopherol-430). Other workers had previously reported isolation of metabolic products of alpha tocopherol which, they indicated, were dimers and trimers. Two previously proposed structures for the dimer had molecular weights of 824 and 856 respectively.

With the availability of the mass high resolution spectrometer, further studies were undertaken to investigate these polymerization products. Polymers of alpha tocopherol were synthesized by chemical oxidation and purified by chromatography. Three main fractions were isolated. Two were shown to be dimers and the third, trimer. Metabolic products were purified in a similar manner and were separated into three fractions. These were shown to chromatograph with the three biologically synthesized fractions.

Extensive studies of the major chemically-synthesized dimer fraction, which relied heavily on high-resolution mass spectrometry (which has not been previously applied to this problem), revealed an unsuspected complexity to the structure. However, it does seem certain that at least one fraction of the dimer isolated by T.L.C. has a molecular weight of 858 and it almost certainly has a free hydroxyl group which has not previously been appreciated.

In order to assess physiological significance of the polymerization, tracer doses of radioactive alpha tocopherol were fed to rats and mice. No polymers could be demonstrated in the liver. Such a finding suggests that polymerization is not a major pathway of alpha tocopherol metabolism except under nonphysiological loading conditions.

Significance to Heart Research: Certain investigators have implied that aging and heart disease are related to alpha tocopherol levels and metabolism.

Proposed Course of Project: No further studies are planned at this time.

Publications: None

814

Serial No.-NHI-294
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:	In Vitro Studies on Phytanic Acid Metabolism
Previous Serial No.:	NHI-278
Principal Investigators:	Su-Chen Tsai, Ph.D. Daniel Steinberg, M.D., Ph.D.
Other Investigators:	G.W.A. Milne, Ph.D. Henry M. Fales, Ph.D.
Cooperating Units:	Section on Chemistry, Laboratory of Metabolism
Man Years:	Total: 1.3 Professional: 1.3 Other: -

Project Description:

Objectives: To study the mechanism of phytanic acid degradation in vitro.

Methods Employed: Subcellular fractions of rat liver were incubated with phytanic acid-U-^{14}C α-hydroxyphytanic acid-U-^{14}C or pristanic acid-U-^{14}C. Oxidation to $^{14}CO_2$ was determined and the lipid products were sub-fractionated using thin-layer chromatography, gas-liquid chromatography and radioassay.

Major Findings: It has been shown that essentially all of the activity for phytanic acid oxidation in rat liver resides in the mitochondrial fraction. The supernatant fraction and the microsomal fraction are without demonstrable activity. Activity recovered in the mitochondrial fraction is actually greater than the activity of the unfractionated homogeny and this is believed to be due to the inhibitory action of microsomes. When microsomes are added to a washed mitochondrial preparation the rate of phytanic acid oxidation is depressed.

Optimal rates of oxidation of phytanate require the addition of ATP, NADPH, Mg^{++} and Fe^{+++}. Addition of coenzyme A is inhibitory but the reasons for this are not established. Ferrous iron failed to substitute for ferric iron and actually ferrous iron is inhibitory. Dipyridil is also inhibitory.

Detailed studies have been done of the degradation products formed during incubation of labeled phytanic acid with mitochondria. Alpha

815

hydroxyphytanic acid has been definitively identified as has pristanic acid and also the next lower intermediate on our proposed pathway (i.e. 4, 8, 12-trimethyltridecanoic acid). These findings add considerable strength to the proposal. The pathway proposed is indeed the major pathway for oxidation of phytanic acid. Alpha hydroxyphytanic acid was also oxidized by mitochondria in the presence of ATP, NAD and coenzyme A. The overall rate of oxidation was considerably slower than that of phytanate but addition of carnitine yielded a ten-fold increase in rate. Addition of ferric iron stimulated markedly. With the addition of all of these cofactors the rate of oxidation of alpha hydroxyphytanate becomes comparable to that for phytanate. Thus it is concluded that alpha hydroxyphytanate is an intermediate although exogenous alpha hydroxyphytanate appears not to penetrate as well as does phytanic acid. The intermediate degradation products formed during incubation with alpha hydroxyphytanate are those discussed above as products of phytanate oxidation, again supporting the position of alpha hydroxyphytanate on the major pathways.

Delta^2-pristenic acid was identified as a breakdown product. Its identification was based on its retention time in gas-liquid chromatography, conversion to 4, 8, 12-trimethyltridecanoic acid by oxidation with ozone and conversion to pristanic acid by hydrogenation. The latter study was actually carried out using duterium gas and mass spectroscopy confirmed both the identification as pristanic acid and the incorporation of duterium in the product analyzed in the mass spectrometer.

The oxidizing system for pristanic acid, like that for phytanic acid, is almost exclusively located in mitochondria. Optimal rates of oxidation required ATP, coenzyme A, NAD fumarate and Mg^{++}. NADPH, ferric ion and carnitine gave little or no stimulation. With optimal fortification as much as 40 to 50% of added pristanate UC-^{14} was converted to $^{14}CO_2$ per hour. When NAD was omitted from the system a much larger yield of Δ^2-pristenic acid was obtained (as much as 60 to 70% of the pristanate). This accumulation of the alpha, beta-unsaturated intermediate is unique and presumably reflects the poorer association of the intermediate with the enzymes for beta oxidation. Whether or not the same enzymes are involved in normal beta oxidation of straight-chain acids and in the beta oxidation of pristanic acid is not yet established.

Thus it appears that metabolism of phytanic acid by mitochondria involves at least two systems. The first is the alpha oxidative system which requires NADPH, Fe^{+++} and oxygen; second is the beta oxidative system which requires ATP, coenzyme A, NAD and fumarate.

Significance to Heart Research: These studies provide important confirmation for the proposed pathway for phytanic acid degradation. With elucidation of the intimate mechanisms of these transformations, it may be possible to apply such information in studies of human tissues. For example, the defect in tissue culture preparations from patients with Refsum's disease should be evaluated in cell-free preparations. Patients with Refsum's disease show nonspecific ECG changes and several of them have died suddenly, presumably from cardiac arrest or arrhythmia. The reasons for this are not known but may relate to derangement in the conduction system.

An understanding of the biochemical basis for this could be important in this particular syndrome and might be of value in guiding studies of other types of arrhythmia or heart block.

Proposed Course of Research: Further studies to define the intimate mechanism, isolate α-oxidative enzymes and study their biochemical characteristics. Comparison of this pathway with that for α-oxidation in nervous tissue and in plants. Studies to establish which isomer(s) of α-hydroxyphytanic acid are formed enzymatically from phytanic acid. Ultimately, to test tissues from patients for levels of the several enzymes involved.

Publications:

Tsai, S-C., Herndon, J. H., Jr., Uhlendorf, B. W., Fales, H. M., and Mize, C. E.: The Formation of Alpha-Hydroxy Phytanic Acid from Phytanic Acid in Mammalian Tissues. Biochem Biophys. Res. Comm., 28: 571-577, 1967.

817

Serial No. NHI-295
1. Laboratory of Metabolism
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:	The Effects of Hormones on the Metabolism of Adipose Tissue studied in Vitro.
Previous Serial No.:	NHI-279
Principal Investigator:	Martha Vaughan, M.D.
Other Investigators:	Sally Stanley and Ferol Lieberman
Man Years:	Total: 2.7 Professional: 0.8 Other: 1.9

Project Description:

Objectives: To investigate the metabolism of adipose tissue in order to elucidate the mechanism by which this tissue regulates triglyceride synthesis and breakdown providing for storage of energy and its release in the form of fatty acids. To study the mechanism of action of the several hormones which apparently participate in the regulation of its metabolism.

Methods Employed: In vitro incubation of free fat cells to assess effects of hormones, nucleotides and other agents on glycerol production. Chromatography of nucleotides and products thereof followed by radioassay (C^{14} or H^3) in these compounds after incubation of labeled cyclic-AMP with fat cells or tissue extracts. Assay of adenyl cyclase activity using ATP^{32} in broken cell preparations to investigate effects of hormones and drugs on this system.

Major Findings:

Effects of Ca^{++}, Mg^{++} and K^+ on lipolysis stimulated by hormones or by cyclic 3',5'-AMP: In order to complete the study begun in the previous year, a few more experiments in this area were carried out. Originally the effects of ions on lipolysis stimulated by epinephrine, theophylline, cyclic-AMP and its dibutytyl derivative were compared. More recently, the influence of ions on the action of glucagon and ACTH were investigated. Glycerol production was measured with several different concentrations of epinephrine, ACTH or glucagon over a wide range of Ca^{++} concentrations from zero to 15 meq/L. Although the concentration of Ca^{++} required for maximum hormone effect was essentially the same for epinephrine and ACTH, with low concentrations of the hormones, omission of Ca^{++} completely abolished the effect of ACTH and decreased that of epinephrine by only about 25%. At very high concentrations of the hormones omission of Ca^{++} was without effect. The effect of glucagon was uninfluenced by Ca^{++} concentration (or its absence).

It was found some time ago that in medium lacking both Ca^{++} and Mg^{++}, stimulation of lipolysis by 3',5'-AMP could be demonstrated. The magnitude of the effect, however, was quite variable. In searching for the cause of this variability, it was found that the addition of either Ca^{++} or Mg^{++} to medium in concentrations only a fraction of that contained in Krebs-Ringer phosphate medium markedly decreased the lipolytic effect of the nucleotide. In the light of this finding, the source of water and some of the reagents used in preparing medium was changed and it now seems possible to consistently demonstrate a sizeable stimulation of lipolysis by 3',5'-AMP (particularly when K^+ also is omitted from the medium, or ouabain is added).

Effects of cyclic 3',5'-IMP (prepared in the laboratory) and 3',5'-GMP (purchased) on lipolysis have been investigated. Either in complete Krebs-Ringer phosphate medium or in the all-sodium medium in which 3',5'-AMP is most effective, no significant stimulation or inhibition has been observed.

Uptake of H^3 or C^{14}-labeled 3',5'-AMP by free fat cells: Our earlier studies had shown that although 3',5'-AMP usually does not stimulate but rather inhibits lipolysis in Krebs-Ringer ph sphate medium, omission from the medium of Ca^{++} and Mg^{++} made it possible to reproducibly demonstrate a lipolytic effect of this nucleotide. Further addition of ouabain or omission of K^+ markedly enhanced this effect. This group of studies was carried out in order to determine whether the effects of these ions on the uptake and metabolism of 3',5'-AMP could be correlated in any way with their influence on the lipolytic action.

Fat free cells were incubated for varying periods of time in medium containing H^3 or C^{14}-labeled 3',5'-AMP. At the end of the incubations, samples of medium and of washed cells were taken for extraction, separation and radioassay of 3',5'-AMP and its products. Most experiments were carried out with 3',5'-AMP at a concentration of 1.5-5.0 x 10^{-6} M in the medium at zero time. Concentrations in this range have not been shown to stimulate lipolysis in fat cells but under appropriate conditions will inhibit lipolysis stimulated by theophylline. A few experiments were done using 5 x 10^{-4} M 3',5'-AMP (a concentration that will stimulate lipolysis in all Na^+ medium). The latter experiments are difficult to interpret quantitatively, since the amount taken up from medium is only a small fraction of the total. In part for this reason, and perhaps for others, it has been difficult to obtain good balances for total radioactivity (i.e. cells plus medium) after one hour of incubation. In the experiments with lower concentrations of 3',5'-AMP in the medium, the radioactivity in cell ATP, ADP, AMP, IMP, 3',5'-AMP, adenosine and inosine usually corresponds closely to the total uptake. In the medium there is little or no detectable appearance of radioactivity in other nucleotides, adenosine or inosine.

The uptake of 3',5'-AMP from concentrations of the order of 10^{-6} M was readily measured and the rate in the first 20 minutes was quite reproducible (mμmoles/g cells) from one batch of cells to another. Omission of Ca^{++} from the medium had no effect on the rate of uptake or on the distribution of radioactivity in cell nucleotides and nucleosides. In medium containing no Mg^{++} the rate of uptake was only 30-50% of that in complete medium. There

819

was, however, no significant change in the distribution of radioactivity in the intracellular products of 3',5'-AMP metabolism. Findings in medium without Ca^{++} and Mg^{++} were similar to those in medium from which only Mg++ was omitted. Omission of K^{+} from medium without Ca^{++} and Mg^{++} had little effect on the rate of 3',5'-AMP uptake, but did markedly decrease the fraction of label from 3',5'-AMP that accumulated in cell ATP, ADP and AMP.

In all experiments, only a few percent of the total cell radioactivity was present in cells as 3',5'-AMP (except in zero time samples in which 40-50% of the small amount of radioactivity present was usually in the form of 3',5'-AMP). The intracellular concentration of labeled 3',5'-AMP was not significantly altered by changing the ionic composition of the medium or by the addition of theophylline in concentrations known to stimulate lipolysis under the conditions employed. It seems clear that the remarkable influence of medium ionic composition on the lipolytic action of exogenous 3',5'-AMP is not explained by changes in the rate at which fat cells take up the nucleotide under these conditions. The observation of the effect of Mg^{++} on 3',5'-AMP uptake may, however, provide a clue concerning the mechanism of uptake -- and perhaps release -- of this and other cyclic nucleotides by cells.

Adenyl Cyclase Activity in Broken Cell Preparations: An assay for adenyl cyclase activity slightly modified from the method of Krishna, using αP^{32}-labeled ATP has been set up. Homogenates of rat adipose tissue were prepared in several different media and fractions separated by centrifugation. Several methods of preparation yielded particles with considerable cyclase activity as assayed in the presence of fluoride, but no effects of hormones were demonstrable with any of these. Effects of substrate concentration, 5'AMP, ADP, an ATP generating system (phosphoenol pyruvate plus pyruvate kinase) and dithiothreitol were investigated with these preparations, but as no effects of hormones were obtained, attention was then directed toward different methods of breaking cells and separating cell fractions.

By first preparing free fat cells, then suspending them in a hypotonic medium without serum albumin, freezing rapidly with dry ice-ethanol and thawing slowly at room temperature, it has been possible to obtain a fraction of particles collected by low-speed centrifugation that responds dramatically to ACTH, epinephrine and glucagon. Adenyl cyclase activity is increased 5-10-fold over the basal level by each of these hormones -- effects that are considerably greater than have thus far been reported. After removal of this fraction, further centrifugation of the supernatant fraction (12,000 x g x 10 min) yields a fraction that also contains adenyl cyclase activity, but which responds little if at all to hormones. The activity per mg protein in this second fraction, assayed with or without NaF is not very different from that in the first fraction.

Under the conditions used, cyclase activity is proportional to the amount of enzyme added. The time course, effects of pH, hormone concentration and NaF concentration have been investigated. Preparations have been stored for up to 8 days at -70° with no significant change in cyclase activity or in response to hormones.

Significance to Heart Research: Because plasma lipid levels and obesity are both highly correlated with incidence of coronary artery disease, basic knowledge concerning factors regulating lipid mobilization must be obtained if we are to fully understand the biochemistry involved.

Proposed Course of Project: Studies of the adenyl cyclase in fat cells will be extended in an attempt to describe, and ultimately to elucidate, the mechanism of the effects of several hormones that act, apparently directly, to stimulate (epinephrine, ACTH, glucagon) or perhaps to inhibit (insulin, PGE_1, acetylcholine) cyclic AMP formation. Studies with other hormones (e.g. triiodothyronine, growth hormone) that may act indirectly on this system will also be explored.

Cyclic-GMP has been reported by Sutherland's group to be present in tissues. The urinary excretion of this nucleotide is said to be influenced by endocrine state. It is planned to set up an assay for GMP cyclase, to attempt to demonstrate the presence of this system in fat cell homogenate fractions and to explore the effects of hormones on it.

It is planned also to return, after a lapse of several years, to studies relating to the mechanism of action of the hormone-sensitive lipase in adipose tissue.

Publications:

Mosinger, B. and Vaughan, M.: Effects of Electrolytes on Epinephrine-Stinulated Lipolysis in Adipose Tissue in Vitro. Biochim. Biophys. Acta, 144, 556-568, 1967.

Mosinger, B. and Vaughan, M.: The Action of Cyclic 3',5'-Adenosine Monophosphate on Lipolysis in Rat Adipose Tissue. Biochim. Biophys. Acta, 144, 569-582, 1967.

821

Serial No. NHI -296
1. Laboratory of Metabolism
2. Section on Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mass Spectrometry and Structure of Natural Products

Previous Serial Number: NHI-280

Principal Investigators: H. M. Fales, Ph.D. and D. Baty, Ph.D. (Visiting Sci. from England)

Other Investigators: P. F. Highet, B.S.

Cooperating Units: None

Man Years:

Total:	1.0
Professional:	0.5
Other:	0.5

Patient Days: None

Project Description:

Combined GLC-mass spectrometry on the LKB spectrometer has proved extremely useful and several new compounds and intermediates have been discovered with its aid. We have found that the LKB can be used with a mixture of compounds to assign peaks of nominal mass to distinct spectra. Mass measurements can then be performed on the mixture using the MS-9 high-resolution spectrometer.

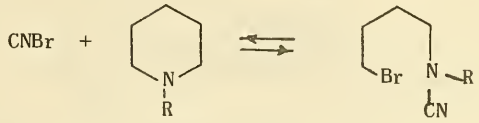
Using this technique, a new N-ethyl derivative of a cactus alkaloid has been isolated - N-ethylanhalonine - and its significance in acetate biosynthesis considered.

A method has been developed for differentiating 1,3- and 1,2-diglycerides via their characteristic cracking patterns in the mass spectrometer.

A phospholipid obtained by B. Prescott (NIAID) from mycoplasma pneumoniae has been investigated in some detail. Attempted purification on silica gel gave material of varying activity and the project has been temporarily suspended.

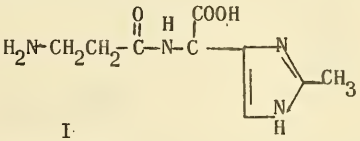
822

The mechanism of the von Braun cyanogen bromide degradation of amines has been studied and the reaction has been found to be reversible.

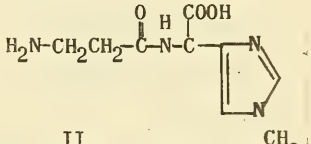


Mass spectrometry shows that halogenated solvents can participate since chloro derivatives were obtained in addition to bromocyanamides. The method thus has synthetic as well as degradative utility.

The structure of ophidine has been revised on the basis of nmr spectroscopy from I to II



I



II

S-Palmitylpantetheine has been found in liver homogenates and its structure elucidated with the MS-9 spectrometer.

Honors and Awards: None

Publications:

Lloyd, H.A., and Fales, H.M.: Terpene alcohols of Helichrysum Dendroideum, Tetrahedron Lett., 48, 4891-4895, Dec. 1967.

Tsai, Su-Chen, Herndon, J. H., Uhlendorf, B. W., Fales, H. M., and Mize, C.E.: The formation of α-hydroxyphytanic acid from phytanic acid in mammalian tissues, Biochem. Biophys. Res. Comm., 28, 571-577 (1967).

Wolff, J., Horisaka, K., and Fales, H. M.: On the structure of ophidin, Biochemistry, In press (1968).

823

Serial No. NHI-297
1. Laboratory of Metabolism
2. Section on Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 30, 1967 through June 30, 1968

Project Title: Aromatic Biosynthesis

Previous Serial Number: NHI-281

Principal Investigators: H. M. Fales, Ph.D. and P. G. Gordon, Ph.D. (Visiting Sci. Australia)

Other Investigators: None

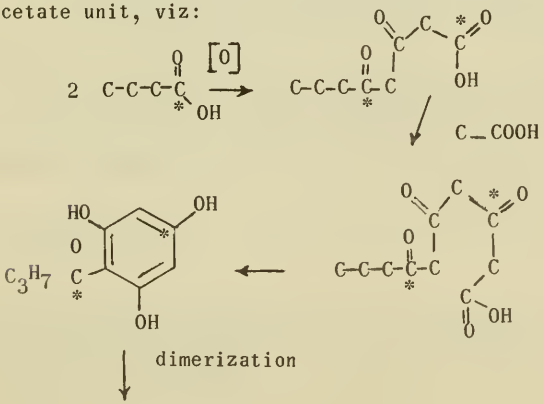
Cooperating Units: None

Man Years: Patient Days:

Total:	1.5
Professional:	1.5
Other	0.0

Project Description:

Previous evidence suggesting intact incorporation of butyric acid into margaspidin (I) in D. marginalis has been confirmed by the degradation outlined below. The results imply that two 4-carbon units have joined head-to-tail to form an octanoate derivative which then adds a 2-carbon acetate unit, viz:



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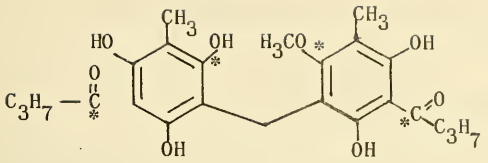
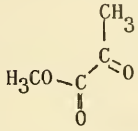
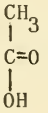
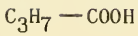


I

ozone



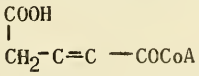




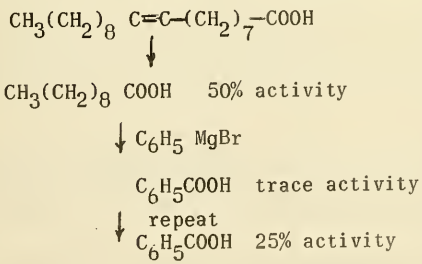
25% activity ~1% 25% activity

The methyl pyruvate was collected by gas chromatography and contained 25% of the activity. Acetic acid contained only a trace of activity, proving that this organism does not efficiently degrade butyrate to acetate.

The suggested intermediate in this unusual coupling reaction is carboxycrotonyl CoA II, a simple vinylogue of malonyl CoA



Oleic acid from the same plant has been degraded as follows:



Ozone also furnishes short chain acids C_9 C_4 and the activities confirm the above pattern, proving that the "4-carbon hypothesis" operates in the

formation of fatty acids also, at least in this organism.

Mammalian systems will be studied next to determine whether this new biosynthetic route is operative.

Honors and Awards: None

Publications:

Gordon, P. G., Penttila, A., and Fales, H.M.: The incorporation of sodium butyrate into methylenebis(butyrylphloroglucinols) by a novel biosynthetic pathway, J. Amer. Chem. Soc., 90, 1376-1377, Feb. 1968.

826

Serial No. NHI-298
1. Laboratory of Metabolism
2. Section on Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Characterization of Natural Materials

Previous Serial Number: NHI-282

Principal Investigator: R. J. Highet, Ph.D.

Other Investigator: Patricia F. Highet, B.S.

Cooperating Units: None

Man Years		Patient Days
Total:	1.5	
Professional:	1.0	None
Other:	0.5	

Project Description:

A. Nuclear Magnetic Resonance Studies of materials of biological origin have been extended by the installation of a Varian Associates HA-100 NMR Spectrometer with spectrum accumulating accessory. Spectra have been obtained from minute samples, as small as a micromole, rendering feasible the study of materials isolated by such techniques of thin-layer and paper chromatography, and gas liquid chromatography. With the supplementary probes in hand, spectra of ^{31}P, ^{59}Co and ^{13}C have been obtained.

A study of nuclear magnetic double resonance (NMDR) as an aid to the characterization of complex molecules has been initiated. Preliminary studies have shown that the nuclear Overhouser effect will provide a valuable aid to the characterization of amaryllis alkaloids.

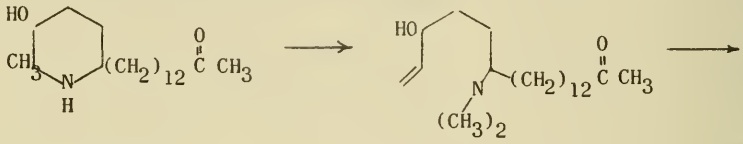
B. Mass spectral studies of sesquiterpenoid lactones related to Helenalin (conducted in collaboration with Dr. Lin Tsai, Laboratory of Biochemistry, NHI and Prof. Werner Herz of Florida State University) have been completed by the elucidation of routes of their fragmentation under electron impact.

An anomalous route for the fragmentation of ω-amino alkyl methyl ketones has been demonstrated by study of representative members, including alkaloids of Cassia excelsa. In the fragmentation of these compounds, the well-characterized McLafferty rearrangement is largely or completely

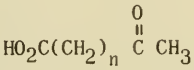
827

suppressed, with the unexpected loss of the acetonyl radical accounting for the major peaks at high mass.

C. Characterization of the alkaloids of Cassia excelsa has proceeded with the elucidation of the structure of casselsine by degradative and spectral studies. Hofmann degradation of N-methylcasselsine provides the olefinic amine II, rather than the anticipated epoxide. A second Hofmann reaction and oxidation produce the ketoacids III and IV, and establish the structure of the parent alkaloid as I



I II

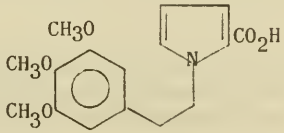


III n = 11

IV n = 12

The alkaloid excelsine has been shown to be dl-cassine, its occurrence implying an unusual enzymatic racemization, or the even more unusual competitive formation of both d- and l-forms of the alkaloid in vivo. Tentative structures have been assigned to three other alkaloids of the plant.

D. Miscellaneous studies have included the characterization of a natural alkaloid of peyote studied in collaboration with Prof. Govind Kapadia of Howard University. Peyonine, a neutral alkaloid, has been shown to possess the structure I.



From the neutral compounds of Millettia ferruginea have been obtained sucrose, and a triterpenoid tentatively identified as taraxerol.

In collaboration with Dr. Ulrich Weiss of the National Institute of

628

Arthritis and Metabolic Diseases, a study of the red pigment of Lachnanthes tinctoria has been initiated.

The Future Course of the Project will extend the studies of NMDR in the characterization of complex materials, and initiate the study of ^{31}P spectra of materials of biological interest. The feasibility of studying the spectra of insensitive nuclei such as ^{13}C by the rapidly developing techniques of pulsed spectrometers will be investigated.

Antibiotics related to oligomycin which are known to inhibit mitochondrial respiration will be studied in collaboration with Dr. G. W. A. Milne of this Section and members of the Laboratory of Chemical Pharmacology.

A study of the biosynthetic origin of terpenoid materials in collaboration with Dr. Jean-Francois Biellmann of the Institut de Chemie of Strasbourg will be initiated in those laboratories.

Honors and Awards: None

Publications:

Kapadia, G. J., and Highet, R. J.: Peyote alkaloids IV; the structure of peyonine, a novel β-phenethylpyrrole from Lophophora williamsii. J. Pharm. Sci. 57, 191

Highet, R. J., Ma, J. C. N., and Highet, P. F.: Dihydrotazettine methine, an unsual non-coplanar phenylcyclohexene. J. Org. Chem. 33, in press (1968).

Highet, R. J., and Highet, P. F.: The absolute configuration of alkaloids related to crinine, tazettine and manthine. J. Org. Chem. 33, in press (1968).

829

Serial No. NHI-299
1. Laboratory of Metabolism
2. Section on Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Characterization of Natural Products

Previous Serial Number: NHI-283

Principal Investigator: Helen A. Lloyd, Ph. D.

Other Investigator: None

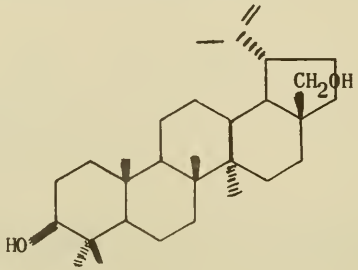
Cooperating Units: None

Man Years		Patient Days
Total:	1	None
Professional:	1	
Other:	0	

Project Description:

The use of high resolution NMR spectroscopy, combined gas chromatography-mass spectrometry, high resolution mass spectrometry and preparative gas-liquid chromatography has permitted structural studies on very small amounts of natural products.

The study of the constituents of the leaves of the Australian compositae Helichrysum dendroideum was continued. A triterpene alcohol, $C_{30}H_{50}O_2$, was isolated and identified as betulin (I). It is noteworthy that this triterpene belongs to one enantiomeric series and the diterpenes isolated from the same plant are of opposite configuration.

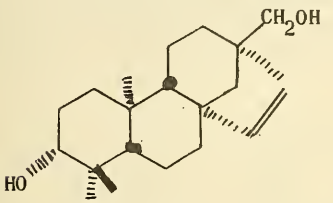


(I)

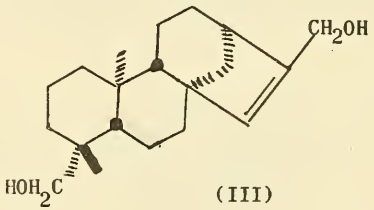
830

This plant is also unusual in that some of the diterpenes constitute hydroxylation patterns of the (+)stach-15—ene nucleus while the others reflect analogous oxidations of the (-)kaur-16-ene system. Although Wenkert had postulated a common intermediate and biogenetic path for the two diterpene skeletons as early as 1955, this is the first time that the coexistence in one plant of kaurene and stachene derivatives has been established.

Eight diterpene alcohols (five isomeric diols, two triols, one tetrol) have been isolated to date from the plant. The structure of three of the diols had been determined earlier as (-)kaur-16-ene-3α,19-diol, (+)stach-15-ene-3α,19-diol and (+)stach-15-ene-17,19-diol. The two other diols have now been fully characterized by nmr and mass spectroscopy and conversion to stachane or kaurane by a series of degradations. They are (+)stach-15-ene-3α,17-diol (II) and (-)kaur-15-ene-17,19-diol (III).

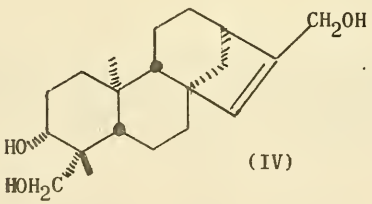


(II)



(III)

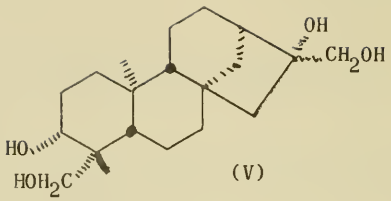
One of the triol has been tentatively assigned structure IV, (-)kaur-15-ene-3α,17,19-triol, an isokaurene allylic alcohol related to diol III.



(IV)

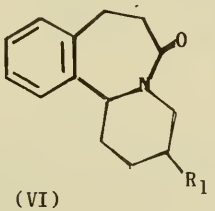
In contrast to the diols ($C_{20}H_{32}O_2$) and triol ($C_{20}H_{32}O_3$), the tetrol ($C_{20}H_{34}O_2$) is saturated. The small amount of material available has precluded a chemical proof of structure at this time; however the nmr and mass spectra point to a 3, 16, 17, 19 kaurane tetrol.

831



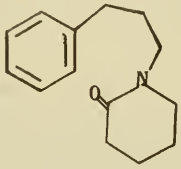
(V)

In connection with the determination of the structure of the alkaloids of Astrocasia phyllanthoides a synthetic study was undertaken. A unique (6,7,6) azatricyclic system, VI, had been postulated earlier for several of these alkaloids. The many attempts to synthesize this novel ring or related systems have been unsuccessful up to this time mostly because of the difficulty of cyclizing intermediates such as VII or VIII to an unactivated benzene ring and also because of unstability of the tricyclic amines formed.

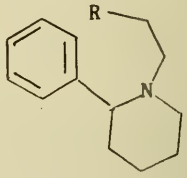


(VI)

R_1 = α-methylpiperidyl

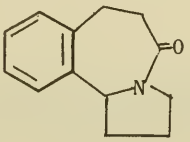


(VII)

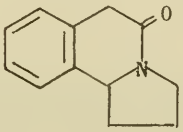


(VIII)

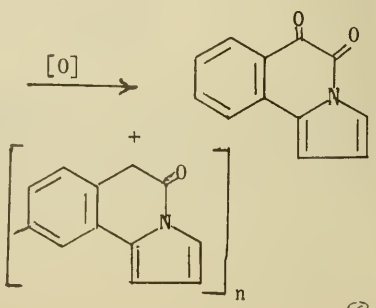
Two other novel tricyclic aza systems were synthesized in good yield by a modified Pictet Spengler synthesis and utilized as IR, nmr and mass spectra models. These lactams are surprisingly unstable and air oxidize in the presence of light to dimers and complex polymers.



(IX)



(X)



The study of the constituents of Helichrysum dendroideum will be continued. With the aid of the LKB combined gas chromatograph-mass spectrometer other mono, di- and triterpene hydrocarbons and alcohols should be identified and lead to an understanding of the biogenetic paths in this plant.

The determination of structure of the minor alkaloids of Astrocasia will be resumed and the synthesis of nitrogenous polycyclic systems related to these alkaloids will be continued.

Honors and Awards: None

Publications:

Lloyd, H. A., and Fales, H. M.: Terpene alcohols of Helichrysum dendroideum. Tetrahedron Letters, 48: 4891-4895, Dec. 1967.

833

Serial No. NHI-300
1. Laboratory of Metabolism
2. Section on Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Use of Digital Computing in Problems in Biochemistry

Previous Serial Number: NHI-284

Principal Investigators: H. M. Fales, Ph.D., G.W.A. Milne, Ph.D., and E. Gilbert, B.S. (CDPB-DCRT).

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.5
Professional:	0.5
Other:	0

Patient Days

None

Project Description:

Approval has been sought and obtained for the purchase of a small computer, to be used on-line with the two mass spectrometers in the laboratory. To this end, an Invitation for Bids has been prepared and published. Meanwhile, the analog data from the high resolution mass spectrometer is being digitized on the hybrid system in the Clinical Center. The resulting digital data is then handled by the IBM-360 system for which the necessary software has been written but not completely debugged.

The mass spectrometer itself has been considerably improved by the block insertion of commercial or NIH-built stabilized power supplies in place of battery-driven amplifiers. Plans are now proceeding to replace the spectrometer's chopper-driven amplifiers with solid state devices which are one fifth as expensive and considerably more reliable.

The low resolution LKB-9000 mass spectrometer has been successfully interfaced with the hybrid computer and will routinely produce bar graph spectra in the near future. The feasibility of accurate mass (and hence formula) determination by this technique is being investigated.

A program in computer-assisted interpretation of spectra has been begun in collaboration with scientists of the Artificial Intelligence Unit of DCRT,

headed by M. Slagle. The ultimate goal of this program is for the computer to reduce spectral data to a unique structural formula. The computer must therefore be programmed to logically and correctly reassemble the molecule from the fragments which it knows from the mass spectrum to be present. Confirmation of its decisions is continually sought by the computer and, wherever possible, made available from, for example, infrared or nmr data.

Related to this above project are plans to convert the MS-9 to a combination electron-impact/chemical ionization mass spectrometer. Chemical ionization is a relatively low energy method of ionizing the molecule under investigation. The structural integrity of the molecule that is ionized in this way is disturbed far less and the potentially available information is increased correspondingly. This combined source has been designed and should become available in the near future.

Honors and Awards: None

Publications: None

835

Serial No. NHI-301
1. Laboratory of Metabolism
2. Section on Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Chemical Investigation of Biochemical Reactions

Previous Serial Number: NHI-285

Principal Investigator: G. W. A. Milne

Other Investigators: None

Cooperating Units: None

Man Years		Patient Days
Total:	0.4	None
Professional:	0.4	
Other:	0	

Project Description:

In collaboration with J. Holtzman and J. Gillette, the mechanism by which naphthalene is converted by liver microsomes to 1,2-dihydro-1,2-dihydroxynaphthalene has been elucidated. Labeling experiments with ^{18}O have shown that one atom of oxygen is inserted from O_2 into the α-position of the substrate and the other atom of oxygen is incorporated from water. The stereochemistry of the resulting diol is trans and with these data, a fairly complete mechanism for this reaction can be written.

The established route for oxygenation of aromatic systems by pseudomonas is considered, by contrast, to proceed via a cis-dihydro diol, and this stereochemistry should be essential to the successful further metabolism of the substrate. A consequence of this hypothesis is that 2-fluorobenzoic acid should be metabolised by pseudomonas to give, in addition to the products reported (NHI,293 1966), catechol. Furthermore the oxygenations of this catechol should both be derived from molecular oxygen. Labelling experiments carried out in collaboration with Peter Goldman and J. Holtzman, using combined gas-chromatography-mass spectrometry have confirmed this theory in every detail. This permits a clearer understanding of the mechanism of the metabolism of anthranilic acid, reported ten years ago by Hayaishi.

In a continuing study with Peter Goldman of the metabolism of aliphatic halogenated compounds, ^{18}O labeling experiments have been carried out with haloacetate, halopropionate and halobutyrate. Walden inversion and incorpora-

816

tion of ^{18}O from $H_2{}^{18}O$ occurs in all these cases. The same micro-organisms fails however to metabolize α-halo-isobutyrate and it seems therefore that the conversion of halo-acid to hydroxy acid follows the surprisingly complex route taken by 4-fluoroproline, which is converted to 4-hydroxyproline in actinomycin biosynthesis.

Work with scientists from LC-NIAMD on the N-bromosuccinimide oxidation of tyrosine derivatives has been completed. The major products derived from such reactions are 6-hydroxyindole,5-bromo- and 5,7-dibromo-6-hydroxyindoles. The significance of these findings with respect to N-bromosuccinimide cleavage of peptides has been noted.

The high resolution mass spectra of the macrolide antibiotics, methynolide, erythronolide-B and fungichromin have been studied. The structure-spectra relationships established for these compounds have shed some light on the structure of the ATPase inhibitor, retamycin. This work is to be continued in collaboration with Drs. R. J. Highet, E. Titus and E. Inturrisi.

In collaboration with Dr. J. Baxter, the metabolism of phytanic acid in rats has been studied. Five isomers of phytanic acids were isolated from rat lymph and their structures were determined by mass spectroscopy and nuclear magnetic resonance spectroscopy.

Honors and Awards: None

Publications:

Holtzman, J., Gillette, J. R., and Milne, G.W.A.: The incorporation of ^{18}O into naphthalene in the enzymatic formation of 1,2-dihydronaphthalene-1,2-diol. J. Biol. Chem. 242: 4386-4387, (1967).

Holtzman, J., Gillette, J.R. and Milne, G.W.A.: The metabolic products of naphthalene in mammalian systems. J. Amer. Chem. Soc., 89: 6341-6344, Nov. 1967.

Goldman, P., Milne, G.W.A., and Keister, D. B.: Carbon-halogen bond cleavage, III. Studies on bacterial halidohydrolases. J. Biol. Chem., 243: 428-434, Jan. 1968.

Wilchek, M., Spande, T.F., Milne, G.W.A., and Witkop, B.: Chemical conversion of tyrosine to mono- and dihydroxyindoles. Biochemistry, 7, 1777-1786, May, 1968.

237

Serial No. NHI-302
1. Laboratory of Metabolism
2. Section on Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Application of Mass Spectrometry to Problems in Biochemistry

Previous Serial Number: NHI-286

Principal Investigator: George W. A. Milne, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years		Patient Days
Total:	0.4	None
Professional:	0.4	
Other:	0	

Project description:

In collaboration with F. Eisenberg, a series of specifically deuterium labeled glucose molecules have been synthesized. The mass spectra of the acetates of these compounds have been determined and in this way, the whole problem of electron impact-induced fragmentation of carbohydrates has been considerably clarified. As a result of this work, location of a deuterium label in a sugar is now routine and the prospects for stereochemical assignments seem better than has previously been considered to be the case.

The mass spectra of a series of small peptides have been studied as part of a joint effort with A. Kiryushkin and Yu. A. Ovchinnikov of the USSR Academy of Sciences, Moscow, USSR. These peptides are unusual in that they appear to undergo intermolecular reactions in the mass spectrometer, giving rise to ions of mass greater than the molecular weight of the peptide. In view of the vigorous work going on in this area, definition of this process is considered of some urgency and work is continuing in this direction.

Work on the anti-radiation compounds related to 2-amino-2-thiazoline is being continued. The differing behavior of this compound towards isocyanates and isothiocyanates has been observed and the structure of the product obtained in either case has been elucidated, mainly by high resolution mass spectrometry.

838

The intriguing chemistry of nitrosamines continues to receive considerable attention. A double-labeling experiment, involving ^{15}N and ^{2}H has been used to establish the intermolecular nature of the photochemical rearrangement of nitrosamines to amidoximes. An anomalous rearrangement process in the mass spectrum of nitrosamines has been clarified as have the remarkably complicated nuclear magnetic resonance spectra of ^{15}N-labeled nitrosamines. Extension of this nmr work to more complex systems is in progress as is a complete study of the infrared spectra of these compounds. Inspection of their Raman spectra is also envisaged.

A comprehensive review of the use of high resolution mass spectrometry in organic chemistry, particularly biochemistry, has been written for Quarterly Reviews.

Honors and Awards: None

Publications:

Axenrod, T., and Milne,G. W. A.: Evidence for the intermolecular mechanism in the photoisomerization of N-nitrosodialkylamines to amidoximes. Tetrahedron Letters, 45, 4443-4445, July, 1967.

Axenrod, T., and Milne, G. W. A.: Electron-impact induced rearrangements of dibenzylnitrosamines. Chem. Comm., 2, 67-68, Jan. 1968.

Milne, G. W. A.: The application of high resolution mass spectrometry to organic chemistry, Quarterly Reviews, 22, 75-93 (1968).

Axenrod, T., and Milne, G.W.A.: Photochemistry of nitrosamines. Mechanism studies, Tetrahedron, In press (1968).

839

ANNUAL REPORT
CLINIC OF SURGERY
NATIONAL HEART INSTITUTE
July 1, 1967 through June 30, 1968

The clinical and laboratory investigations carried on in the Clinic of Surgery centered upon those forms of congenital and acquired cardiac disease which are or may become amenable to operative treatment. Many studies have been concerned with prosthetic cardiac valves. In the laboratory, it was found that warfarin did not prevent the formation of tissue on fabric-covered valves, but made the original platelet-fibrin deposit thin, and the final tissue layer was delicate and transparent. Anticoagulants will be given to patients with covered valves for six months after implantation. Covered valves have been utilized in approximately 50 patients in both the mitral and aortic positions. Preliminary hemodynamic data indicate that the original covered mitral valves are unsuitable for further patient use because they are relatively stenotic. An improved mitral prosthesis with a larger orifice and longer path of ball travel is presently being utilized. The Model 2300 aortic prosthesis has been inserted in 25-30 patients, and anticoagulants have been discontinued. There have been no emboli. Preliminary hemodynamic data indicate that this valve may also be stenotic, and a revised version will be made.

Detailed evaluations of the results of aortic valve replacement in 175 patients have been completed. Total early and late mortality with the operation has been 35 percent, but surviving patients have evidenced excellent clinical and hemodynamic improvement. Late death from degeneration of the silastic poppet is a present concern, but one which can be obviated with the valves currently in use. Preoperative findings indicating a high operative risk could not be identified. Thus, coronary artery disease, previous myocardial infarction, age, pulmonary disease, etc. did not increase total risk.

In the laboratory, prosthetic mitral valves of discoid configuration have been covered with fabric and implanted. In every instance the disc wore through the fabric on the struts, indicating that this type of valve covering is probably impractical. Further studies are necessary, however, to determine this point with certainty, since disc-type low profile valves must continue to be used in certain patients in whom the left or right ventricular cavity is unusually small.

Flow meters have been constructed within the orifices of prosthetic valves of both the ball and disc types. These instruments provide a means of measuring instantaneous flow through the valve, and for evaluating valves of different configurations. The pattern of instantaneous flow through a prosthetic mitral or tricuspid valve is normally biphasic. The original peak occurs early in diastole, when filling of the ventricle is rapid. A late diastolic peak in flow then occurs as a result of atrial contraction. At progressively more rapid heart rates the contribution made by atrial contraction diminishes, as does total forward flow per cycle. The volume of reverse or regurgitant flow, which occurs ahead of the seating ball, is constant and is 5-12% of flow

volume at normal rates. With faster heart rates, however, the forward flow per cycle decreases, and the percentage of regurgitant flow increases strikingly. Thus, the data to date incidate that the ball-valve does not operate effectively at rapid heart rates, that regurgitation increases with rising rate, and that flow through the valve is improved by an effective atrial contraction.

Clinical studies have also been made of the results of mitral valve operations. The success or lack of success of cardioversion was assessed retrospectively in 100 consecutive patients in whom mitral valve replacement was carried out. All patients (25%) who were in normal sinus rhythm before operation maintained normal rhythm afterward, although a number had atrial fibrillation for a brief period immediately after operation. In patients with atrial fibrillation before operation it proved impossible to define the duration of fibrillation. The success of long-term restoration of sinus rhythm by DC shock was related to the size of the left atrium and the mean left atrial pressure recorded six months after operation. If the left atrium is small or only moderately enlarged and the left atrial pressure normal, successful and sustained restoration of normal sinus rhythm after mitral valve replacement can be anticipated. If the left atrial pressure is abnormal and residual left atrial enlargement is prominent, an attempt at cardioversion is probably not indicated since the chance of success is small. In 300 patients subjected to mitral commissurotomy clear hemodynamic evidence of recurrent stenosis of the valve after an effective commissurotomy has been documented in 11 patients. Previously it has been considered that stenosis seen postoperatively was always the result of an inadequate operation, rather than a recurrence of the stenosing process in a valve with an adequate orifice. In 50 patients who had left atrial thrombus at operation none had an intraoperative embolus, yet 17 died in the postoperative period, 6 of cerebral emboli. Among 17 patients who received dextran or heparin in the early postoperative period none had emboli. Thus, whenever atrial thrombus is encountered at operation anticoagulation should be initiated early in the postoperative period, and hemodilution should probably be maintained as well.

Postoperative bleeding remains an important complication of open cardiac operations and increased fibrolytic activity has been incriminated as a major cause. In other centers, in fact, fibrinolysis has been found so commonly that routine administration of epsilon-amino caproic acid (EACA) has been advised after every heart operation. This drug may be dangerous, however, since an hypercoagulable state and widespread intravascular thrombosis may result. Detailed hemolytic studies in 25 patients here indicated transient activities of fibrinolysis but normal values had returned by the end of the operative procedure. Progressive declines in the concentration of factor V and platelets occurred for the first 24 hours. There was no evidence that EACA should be given unless a clear and sustained increase in fibrinolysis is shown. Among 700 patients, second operations for the control of bleeding were required in 78. In only 6 was any abnormality of clotting documented. In 45 patients a discrete source of bleeding was identified while in the others it was diffuse. Seventy-six of the 78 patients survived.

Implantation of the internal mammary artery into the wall of the left ventricle is being carried out in large numbers of patients with angina pectoris at many clinics in this and other countries. In this laboratory physiologic

studies related to revascularization of the ventricle have been made. Left ventricular function was assessed in dogs before and after the creation of myocardial tunnels as used in mammary artery implantation, and creation of the tunnels caused no impairment of function. Epicardiectomy, often a part of the operative procedure was found to improve ventricular function as assessed by function curves, dp/dt, and force-velocity relationships. Epicardiectomy also had a protective effect against ventricular fibrillation after coronary artery occlusion. The mechanism by which epicardiectomy improved ventricular performance and rendered the heart less susceptible to ventricular fibrillation is not known with certainty. The terminal sympathetic fibers of the heart lie immediately beneath the epicardium in a loose plexus, and epicardiectomy may, in fact, be a form of sympathectomy. Epicardiectomy has been thought to increase the diastolic volume (compliance) of the ventricle, but it was found to be unaltered in other studies of pressure-volume relationships after the procedure.

Many patients might benefit from a slower but variable heart rate, and attempts to produce a slow rate were made. In 15 dogs the SA node was excised, and nodal rhythm at a slow rate appeared. After 2-7 months, however, sinus rhythm at a normal rate returned. It appeared that a new atrial pacemaker focus will replace the SA node if this structure is destroyed or removed.

A catheter flow meter (velocitometer) was utilized to assess the severity of aortic regurgitation in dogs. An excellent correlation between the regurgitant flow as measured with the catheter and with a standard flow meter on the aorta was found. Flow in pulmonary veins was also measured with a flow transducer inserted into the lumen of one or more veins. Reverse flow resulted with pulmonary artery occlusion, bronchial obstruction and mitral regurgitation.

Several clinico-anatomic correlative investigations emerged from the Pathology Section of the Surgery Branch. Continued efforts have been made to study anatomically the hearts of all patients who died following replacement of one or more cardiac valves. It has become apparent that the initial technical problems involved in the insertion have, for the most part, been eliminated, and the immediate operative mortality has become low. What period of survival may be expected for these individuals remains in doubt. Degeneration of the silicone rubber of the aortic prosthesis is becoming a major problem. Among 49 patients who died one month or later following valve replacement with Starr-Edwards prostheses, 11 died because of severe degeneration of the silicone rubber balls. The intervals between operation and death in each of the 11 patients was 24 months or over. Six had atrophied, fractured and dislodged aortic balls, and five had balls which had swollen and become impacted in the prosthetic cage. Eight of the 11 died suddenly.

In studying the hearts of patients who died following replacement of one or more cardiac valves with prostheses, it was observed that endocardial fibrosis occurs not infrequently in the left ventricle following mitral valve replacement. Of 16 patients who died in the late postoperative period following mitral replacement, extensive endocardial fibrosis was found in 14

of them. In 2 of the 14 patients it was convincing at necropsy that death in them resulted from endocardial fibrosis, which was not associated with coronary arterial narrowing. The cause of endocardial thickening was believed to be turbulence produced within the left ventricular cavity by the caged-ball mitral prosthesis.

Several studies of valve lesions in unoperated patients were also performed. Calcific pulmonic stenosis was observed in seven patients, and was found to occur only in adults and only in those with severe congenital pulmonic stenosis.

In previous studies from this clinic pure mitral regurgitation was found to be the result of rheumatic heart disease in approximately 50% of patients, and to be of nonrheumatic etiology in the other 50%. There are still some patients in the nonrheumatic group in whom it has not been possible to determine an etiologic diagnosis despite extensive clinical and anatomic studies. Two older patients, aged 60 and 84 years, respectively, were studied. Each had severe mitral regurgitation and huge left atrial cavities. The mitral valves at autopsy were normal, however. One had severe scarring of the papillary muscles, but the coronary arteries were normal.

Among 371 patients with severe valvular heart disease studied at necropsy, two were found to have pure acquired tricuspid regurgitation. It is suggested that infective endocarditis is possibly the only cause of isolated acquired tricuspid regurgitation. Each of the two patients described had infective endocarditis on previously normal valves and each was a heroin addict.

For several years the heart has been known to be involved in patients with rheumatoid arthritis. Accurate descriptions of where rheumatoid nodules occur in the heart have been lacking. Two patients were studied in whom rheumatoid nodules occurred in each of the four cardiac valves. The locations of the nodules in the valves were found to be highly specific, and the valvular disease seen in this entity is pathognomonic.

Since 1960 it has been known that fibrosis of the left ventricular muscles may cause mitral regurgitation. By far the most common cause of this localized fibrosis is coronary arterial narrowing. A 39-year-old patient was studied in whom, at autopsy, the left ventricular papillary muscles were scarred and the coronary arteries were normal. Clinically, the patient had had mitral regurgitation and a large heart apparently on the basis of primary myocardial disease. This patient was the first reported with papillary muscle fibrosis causing mitral regurgitation in primary myocardial disease.

Since idiopathic hypertrophic subaortic stenosis was described in 1958, a number of reports have emphasized the features of this entity. Most of the patients have been relatively young. A patient was studied who had the clinical and anatomic features of this entity and yet was 74 years of age. This was the oldest patient reported with IHSS.

A patient studied with tetralogy of Fallot was found to have severe aortic regurgitation. This patient stimulated a review of the aortic valves in 45 previously autopsied patients with tetralogy of Fallot. Five were found to have anatomic malformations of the aortic valve, and in two the anomalies were of functional importance.

A 40-year-old patient with the Taussig-Bing complex and severe arterial oxygen desaturation was studied. Not only did the patient live 40 years, but at autopsy the coronary arteries were found to be aneurysmally dilated. It was speculated that the aneurysmal dilatation resulted from the severe arterial oxygen desaturation (47%).

It has been known for sometime that conditions which temporarily increase the speed of blood through the heart or great vessels are usually the cause of a transient precordial murmur occurred, but there was no evidence that the speed of flow through the heart was increased. At autopsy an anomalous band was found within the left ventricular cavity extending from the ventricular septum to the lateral free wall. It was speculated that in this patient the systolic murmur occurred when the left ventricle was stretched by retention of fluid, but when the heart failure disappeared, the left ventricular cavity shrank in size and the anomalous band became lax and the murmur disappeared. Anomalous bands as a cause of precordial murmurs had not been previously described.

Calcium is frequently observed in the heart, but mainly in coronary arteries or in valves. A patient was studied with mild right ventricular infundibular obstruction in whom 11 calcified stones were found at autopsy in the right ventricle. The cause of these rocks in the heart was not determined, but similar patients were not found in reviewing the medical literature.

Diffuse disease of the endocardium and of the myocardium has received particular attention in recent years. Three patients were referred to the National Cancer Institute because of "eosinophilic leukemia." Each died of congestive heart failure within 26 months of the onset of his illness. Necropsy disclosed that eosinophilic leukemia is virtually identical morphologically with Löeffler's fibroplastic parietal endocarditis and endomyocardial fibrosis, and demonstrated that myocardial lesions may also occur in this entity.

Since 1936 it has been appreciated that anatomic changes occur in the lungs of patients with severe pulmonary hypertension. A change, however, that has not been previously described in patients with pulmonary hypertension were granulomas containing acicular crystals. During a 12-year period these granulomas were observed in the lungs of 12 autopsied patients. Each had severe pulmonary hypertension. During the same 12-year period, similar pulmonary granulomas were not observed in any patient studied at autopsy who did not have pulmonary hypertension. By x-ray diffraction the pulmonary crystals were found to consist of cholesteryl palmitate and/or stearate.

Studies of the heart in various neoplastic diseases were performed. The hearts of 196 patients with malignant lymphoma were studied at autopsy and 48 (24%) had lymphoma involving the heart. In 27 subjects the cardiac

lymphoma was observed on gross examination, and in the other 21 patients cardiac lymphoma was found only on the study of histologic sections of heart. Lymphoma in the heart occurred most frequently in mycosis fungoides (33%), and least frequently in Hodgkin's disease (16%). Cardiac lymphoma was found in approximately 25% of the patients with lymphosarcoma, reticulum cell sarcoma, and undifferentiated or mixed cell malignant lymphoma.

Angiosarcoma is a rare malignant tumor which occurs most commonly in the skin and subcutaneous tissue, but may arise from any blood vessel. A 35-year-old woman was studied in whom angiosarcoma originated from the heart. A review of previous patients with this tumor of the heart disclosed that it generally causes obstruction to the right-sided cardiac chambers by hemopericardium with tamponade, by constriction of the ventricles from the epicardial tumor, by vena caval or tricuspid valvular obstruction, or by a combination of these factors.

Studies of heart disease in various animals have continued. The hearts of two calves were studied in which the mitral valves consisted of two equal-sized orifices. A review of the literature showed that this entity, "double orifice mitral valve," has been reported in at least 39 human beings and these studies demonstrated that double orifice mitral valve, although a striking anatomic finding, produces no signs or symptoms of cardiac dysfunction. Unless there are associated defects, this valvular abnormality is compatible with a normal life expectancy.

From the histology laboratory of this section techniques used to obtain large sections of great benefit in studying the heart were described.

Serial No. -NHI-303 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Myocardial Function after Prosthetic Valve Replacement

Previous Serial Number: None

Principal Investigators: Dean T. Mason, M. D.
R. Darryl Fisher, M. D.

Other Investigators: Andrew G. Morrow, M. D.
Eugene Braunwald, M. D.

Cooperating Unit: Cardiology Branch, NHI

Man Years
Total: 1
Professional: 8/12
Others: 4/12

Project Description: Disability in patients with acquired valvular heart disease is generally attributed to the hemodynamic abnormalities which result from the mechanical dysfunction of the affected valves.

Left ventricular function was assessed postoperatively at left heart catheterization in 30 patients in whom Starr-Edwards prostheses had been inserted. It was found that underlying myocardial disease or coronary arterial disease contributed to the preoperative disability in most patients, and may limit complete symptomatic recovery following operation. It was found that the residual impairment of myocardial performance observed in these patients was more pronounced in those in whom isolated mitral or combined mitral and aortic valve replacement had been performed.

In an additional group of 40 patients who had cardiac indices less than 2.25 L./min./M.^{2} and underwent isolated aortic valve replacement, 12 died. The remainder have made marked clinical improvement. Twenty-six of these patients have undergone postoperative catheterization and all showed improvement in their hemodynamic status. It is clear that in patients with isolated aortic valve disease and severe left ventricular dysfunction, satisfactory symptomatic and hemodynamic improvement can be expected.

Proposed Course: Manuscript is now in preparation and the reults of the study are utilized in recommending operation to patients fulfilling these criteria.

Serial No.-NHI-304 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Results of Aortic Valve Replacement in 175 Patients Including Preoperative and Postoperative Hemodynamic Assessments

Previous Serial Number: None

Principal Investigators: R. Darryl Fisher, M. D.
Douglas M. Behrendt, M. D.

Other Investigator: Andrew G. Morrow, M. D.

Man Years
Total: 2
Professional: 1½
Other: ½

Project Title: Since February 1963 isolated aortic valve replacement with the Starr-Edwards prosthesis has been performed in 175 patients. Before operation in each of the 175 patients detailed clinical and hemodynamic assessments were undertaken. Following operation all surviving patients were re-evaluated and in 100 of them hemodynamic studies were performed.

Operative mortality for this group of patients was 14%, and the late mortality has been an additional 21%.

Postoperative cardiac catheterizations have revealed restoration of nearly normal hemodynamics in most patients. The results of operation and events in the postoperative convalescence were subjected to statistical analysis to determine the significance of individual prognostic factors. It was found that a previous thoracotomy or prosthetic valve replacement adversely affected the operative mortality, but no significant factors could be defined for the long term survival rate.

Proposed Course: Manuscript is in preparation and the results are being utilized in evaluating individual patients for aortic valve replacement.

Serial No.-NHI-305 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Postoperative Hemorrhage Following Open Heart Operations: Review of 700 Patients.

Previous Serial Number: None

Principal Investigators: Lynn Peterson, M. D.
R. Darryl Fisher, M. D.
Rudolf N. Staroscik, M. D.

Other Investigator: Andrew G. Morrow, M. D.

Man Years

Total:	1½
Professional:	1½
Other:	0

Project Description: Postoperative intrathoracic hemorrhage following cardiac operations may be related to inadequate surgical hemostasis, excess heparinization, or alterations in the coagulation mechanism. In an attempt to determine the incidence of excessive postoperative bleeding and factors contributing to hemorrhage, the records of more than 700 patients undergoing cardiac operations with cardiopulmonary bypass were reviewed.

Seventy-eight patients underwent a second operation following an initial cardiac operation in which extracorporeal circulation was employed. Forty-nine (63%) of this group were operated upon as semi-emergencies in an attempt to control bleeding and to relieve a deteriorating hemodynamic condition. Twelve hours was the average time before re-exploration, and the average blood loss was 150 cc./Kg. before reoperation. A defect in the clotting mechanism was documented in six patients.

At the time of reoperation, a discrete source of bleeding was found in 45 of 78 patients (58%). Seventy-six of the 78 patients survived the operative procedure; two patients died of ventricular arrhythmias during reoperation.

The postoperative course of this group of patients will be compared with that of a similar group of patients with excessive hemorrhage but not re-explored. A method of management of the patient requiring re-exploration will be presented.

Proposed Course: A method of operative and anesthetic management of the patient requiring reoperation for persistent postoperative hemorrhage will be defined from the results of this study. Manuscript is in preparation.

Serial No. -NHI-306 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Results of Operation in 50 Patients with Left Atrial Thrombi

Previous Serial Number: None

Principal Investigators: Lynn Peterson, M. D.
R. Darryl Fisher, M. D.

Other Investigators: Robert L. Reis, M. D.
Andrew G. Morrow, M. D.

Man Years

Total:	1
Professional:	1
Other:	0

Project Description: With the development of cardiopulmonary bypass the intraoperative mortality from cerebral embolism in patients with left atrial thrombus has been reduced. The purpose of this project was to make a detailed assessment of the results of open heart operation and the post-operative management in patients with left atrial thrombi.

Fifty patients had a left atrial thrombus at operation. There were no documented intraoperative cerebral emboli, yet the mortality was 34% and the major cause of death was a cerebral embolus in the early postoperative period (six patients). A group of 17 patients who had received low molecular weight dextran or heparin within the first 12 hours postoperatively had no emboli; 30 patients received no antithrombotic agents, and there were nine with emboli.

Despite the use of cardiopulmonary bypass there is still a significant risk of systemic embolism in patients who have a left atrial thrombus removed at operation. The use of prophylactic antithrombotic agents has apparently provided protection from a postoperative cerebral embolus.

Proposed course: Manuscript is in preparation.

Serial No. NHI-307 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Restoration of Sinus Rhythm after Mitral Valve Replacement: Correlations with Left Atrial Pressure and Size.

Previous Serial Number: None

Principal Investigators: R. Darryl Fisher, M. D.
Dean T. Mason, M. D.

Other Investigator: Andrew G. Morrow, M. D.

Cooperating Units: Cardiology Branch, NHI

Man Years

Total:	6/12
Professional:	3/12
Others:	3/12

Project Description: Factors relating to successful and sustained restoration of normal sinus rhythm (NSR) were examined in 100 consecutive patients after mitral valve replacement. It was found that elevated left atrial pressure and increased left atrial size both militate against the chronic establishment of NSR by either electrical cardioversion or by spontaneous reversion. The study indicated that following mitral valve replacement, permanent maintenance of NSR can be anticipated only in patients in whom the mean left atrial pressure is normal and in whom left atrial enlargement is only moderate.

Proposed Course: The present findings are utilized in recommending electrical cardioversion to patients following mitral valve replacement.

Publication:

Fisher, R. D., Mason, D. T., and Morrow, A. G.: Restoration of sinus rhythm after mitral valve replacement: Correlations with left atrial pressure and size. Circulation Supp. II-37:173-177, April 1968.

Serial No.-NHI-308
1. Clinic of Surgery
2. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Tissue Ingrowth and the Rigid Heart Valve: Review of Clinical and Experimental Experience During the Past Year

Previous Serial Number: None

Principal Investigator: Nina S. Braunwald, M. D.

Other Investigator: Andrew G. Morrow, M. D.

Man Years
Total: 1½
Professional: 1
Other: ½

Project Description: Previous studies in this laboratory indicated that a porous fabric lattice covering, which encouraged rapid tissue ingrowth, significantly decreased the incidence of thrombus formation on rigid prosthetic heart valves. Since February 1967, ball valves totally covered with fabric have been utilized clinically for replacement of the mitral and/or aortic valves. All aortic prostheses, and recently available mitral prostheses, have also had hollow metal poppets.

Experience to date indicates the following:

1) Some increase in tissue buildup has been noted on the outside of the frame beyond one year, but this has never interfered with valve function. Apparently the high velocity of flow as well as the constant ball motion continue to keep the tissue layers thin on the inflow orifice and inside of the struts.

2) Anticoagulants have not been demonstrated to be harmful to the development of the tissue layers, and even appear to be beneficial by making the initial platelet-fibrin deposit thinner, and thereby accelerating the completion of the tissue encapsulation.

3) The time table for the development of complete autologous tissue layers is slower in man than in either the growing calf or adult dog, and anticoagulation for at least six months after operation is probably desirable.

4) The use of poppets made of hard nonelastomeric materials does not necessarily produce an unacceptable inbalance between tissue buildup on the valve and fabric wear in prostheses of the ball-valve design.

5) Preliminary postoperative hemodynamic data indicate that the Model 2300 aortic prosthesis may, in some patients, be or become stenotic. Modification of the fabric covering and/ or the frame of this prosthesis may become necessary.

Serial No. -NHI-309
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Development of an Autogenous Tissue Covering on Prosthetic Heart Valves: Effect of Warfarin and Dextran

Previous Serial Number: None

Principal Investigator: Nina S. Braunwald, M. D.

Other Investigator: Hamner Hannah, M. D.

Man Years
Total: 1
Professional: ½
Other: ½

Project Description: Previous investigations in this laboratory demonstrated that covering rigid prosthetic heart valves with dacron fabric allowed autogenous tissue ingrowth with subsequent inhibition of valvular thrombosis. However, it seemed probable that Warfarin and Dextran should be administered during the period between valve insertion and completion of tissue ingrowth, a period when thromboemboli might occur. This project was designed to ascertain whether Dextran or Warfarin interfered with autogenous tissue formation, since the process is probably a form of controlled thrombosis.

Dacron-covered, Starr-Edwards prosthetic valves were inserted in the tricuspid position in ten Holstein or Angus calves weighing 65 to 85 kg. Five animals received no postoperative anticoagulants, and were sacrificed at 7 - 20 days. The other five animals received low molecular weight Dextran, 500 cc. (50 Gm.)/day as a 30 minute infusion, for three days; they also received 25 mg. Warfarin intramuscularly on the first postoperative day and 10 mg. intramuscularly each day thereafter; animals in this group were sacrificed at 13 - 37 days.

The prosthetic valves were inspected for gross thrombosis and the dacron fabric was removed from the valve struts and fixed in formalin at the time of sacrifice. Fabric tubes were sectioned, stained with hematoxylin and eosin, and examined microscopically.

Both gross and microscopic examinations disclosed that autogenous tissue covered the prosthetic valves in both control and anticoagulated animals, but that the tissue layer in the anticoagulated group was thinner and more uniform than in the control group.

Serial No. -NHI-309
1. Clinic of Surgery
2. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Dextran and Warfarin do not delay and may architecturally enhance the formation of an autogenous tissue layer on dacron-covered, prosthetic heart valves two to four weeks following tricuspid valve replacement in calves. The use of these agents to prevent emboli during the period between valve insertion and tissue ingrowth seems appropriate.

Proposed Course: Project completed. Submitted to Annals of Surgery for review.

Serial No. -NHI-310
1. Clinic of Surgery
2. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Evaluation of Lens Wear in Cloth Covered Ventricular Prostheses

Previous Serial Number: NHI-98

Principal Investigators: Don E. Detmer, M.D.
James C. A. Fuchs, M. D.
Nina S. Braunwald, M. D.

Man Years
Total: 2
Professional: 1
Other: 1

Project Description: Previous work in this laboratory indicated the desirability of covering rigid intracardiac prostheses with porous fabric. This allowed autogenous tissue covering which markedly reduced the incidence of thrombosis. Sustained friction from the more abrasive configuration of the fabric covered struts produced appreciable wear of soft plastic lenses in "low profile" prostheses which seemed to be less ideally suited to the fabric covering modification than did the ball valve design in which evidenced no wear of the moving part.

In an attempt to modify the low profile valve design to reduce lens wear in a fabric covered valve, the tricuspid valves were replaced with Cross-Jones valves with lenses of Titanium, polypropylene, or Kel F in 21 calves. Sixteen valves have been examined from 2 weeks to 8 months postinsertion. The other calves are well and will be sacrificed at appropriate intervals. Slight notching of the polypropylene and Kel F lenses was noted at four months, while the metal lens exhibited no wear at six months. Biconcave metal lenses wore the struts significantly while biconvex metal lenses did not. Fabric was worn from the inner aspects of the struts in nearly all valves, and this area was left bare initially in some valves as a solution to this problem. Allowing the lens to wear the fabric off the struts proved most satisfactory. Less lens wear was noted with polypropylene and Kel F than was noted with silastic lenses, and biconvex titanium lenses also looked very promising as an answer to protection of the low profile valves by encouragement of nonthrombogenic tissue covering the frame.

Proposed Course: The remaining animals will be followed and sacrificed within one year to determine the amount of wear of the lens and struts with the various materials tested. More polypropylene and possibly titanium lenses will be studied. Collaboration in progress with engineers will permit further modifications of the low profile valve designs in a continued attempt to make them suitable for the future covering modification.

Serial No.-NHI-311 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effect of Open Heart Operations on the Coagulation Mechanism

Previous Serial Number: None

Principal Investigator: R. Darryl Fisher, M. D.

Other Investigators: Andrew G. Morrow, M. D.
H. Grolnick, M. D.

Cooperating Unit: Department of Hematology, The Clinical Center

Man Years
Total: 9/12
Professional: 3/12
Other: 6/12

Project Description: Enhanced fibrinolysis frequently occurs during the course of open heart operations and extracorporeal circulation. This alteration in fibrinolysis activation, as well as other disorders of coagulation, has been incriminated as a cause of excessive postoperative hemorrhage.

Twenty-five randomly selected patients undergoing open heart operations were selected, and detailed hematologic determinations were performed in each before, during, and after operation. Factor V, factor VIII, fibrinogen, plasminogen, prothrombin, platelets, partial thromboplastin, euglobulin clot lysis, and thrombin time were determined at intervals.

It was found that a transient activation of fibrinolysis occurred after sternotomy or thoracotomy in over 3/4 of the patients. However, normal values were present by the termination of extracorporeal circulation in all patients. Progressive declines in factor V and platelet counts were seen in each patient.

Proposed Course: Manuscript is in preparation. The results of the study allow more meaningful interpretation of the occurrence of fibrinolysis during open heart operations and enable a more reasonable use of inhibitors of fibrinolysis activation, e.g. epsilon amino caproic acid.

Serial No. NHI-312 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Current Results of Operative Treatment in Congenital Aortic Stenosis.

Previous Serial Number: None

Principal Investigators: Dean T. Mason, M. D.
R. Darryl Fisher, M. D.

Other Investigators: Andrew G. Morrow, M. D.
Eugene Braunwald, M. D.

Cooperating Units: Cardiology Branch, NHI

Man Years
Total: 9/12
Professional: 6/12
Other: 3/12

Project Description: Forty-five young patients underwent operative treatment for discrete aortic stenosis between 1961 and 1967. Clinical and hemodynamic assessments were undertaken before and after operation in each patient.

Twenty-six patients underwent operation for relief of discrete valvular aortic stenosis. Thirteen patients had discrete subvalvular aortic stenosis, three patients had combined subvalvular and valvular stenosis, and three patients had supravalvular stenosis.

Forty-one of the 45 patients are living. Of the four deaths, one was an operative death and three were late deaths. Of the surviving 41 patients, all but four are asymptomatic. Two of these four patients have residual aortic stenosis, and two have severe aortic regurgitation.

Thirty-four of the surviving 41 patients have returned for postoperative cardiac catheterization. A substantial reduction in the left ventriculo-aortic gradient was achieved in each patient, and in all but five patients the gradient was less than 50 mm. Hg postoperatively. An occasional patient was found to have temporary residual left ventricular outflow obstruction of a moderate degree on the basis of hypertrophic obstruction which was secondary to the prolonged fixed aortic stenosis.

The information provided by the present series of patients indicates that in all the various forms of congenital aortic stenosis, effective relief of obstruction to left ventricular outflow can be accomplished. The

operative mortality is minimal and the postoperative clinical and hemodynamic results are gratifying. However, the ultimate clinical and hemodynamic fate of this group of patients must await continued observation.

Proposed Course: Manuscript is in preparation and additional hemodynamic study of this group of patients is contemplated.

Serial No. NHI-313 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Recurrent Mitral Stenosis after Mitral Commissurotomy: A Hemodynamic Study.

Previous Serial Number: None

Principal Investigators: R. Darryl Fisher, M. D.
Dean Mason, M. D.

Other Investigator: Andrew G. Morrow, M. D.

Cooperating Unit: Cardiology Branch, NHI

Man Years:
Total: 6/12
Professional: 3/12
Other: 3/12

Project Description: Recurrent mitral stenosis after mitral commissurotomy is a term describing the clinical syndrome that occurs occasionally several years after apparently satisfactory mitral commissurotomy. However, no detailed hemodynamic studies are available which clearly demonstrate the recurrence of mitral stenosis after mitral commissurotomy.

Review of the clinical records and catheterization findings of over 300 patients who underwent mitral commissurotomy at this institution was performed. Eight patients were found in whom hemodynamic data clearly demonstrated the progression of mitral stenosis after a satisfactory commissurotomy was done.

The average preoperative left atrial mean pressure was 31 ± 3 mm. Hg, and the average mean left ventriculo-atrial gradient before operation was 21 ± 2 mm. Hg. Marked pulmonary hypertension (average 60 ± 5 mm. Hg) was present in all patients.

Six months after commissurotomy the mean left atrial pressure had decreased in each patient, and there was a statistically significant reduction in the average mean left atrial pressure. The diastolic gradient between the left atrium and the left ventricle had been abolished in half the group and only small gradients remained in the other patients.

However, 4 to 11 years after operation, symptoms recurred in each patient, leading to a second operation on the mitral valve. Cardiac catheterization shortly before the second operation revealed recurrence of mitral stenosis in each patient, as evidenced by a significant diastolic left ventriculo-atrial gradient, pulmonary hypertension, and left atrial hypertension. Mitral

regurgitation was absent as indicated by normal left ventricular cine-angiograms or normal left ventricular dye curves.

Proposed Course: This study offers evidence for a hemodynamic cause for the occasional patient who exhibits progressive cardiac symptoms years after a satisfactory commissurotomy. Manuscript is in preparation.

Serial No. -NHI-314
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Comparison of the Hemodynamic Consequences of the Disc and Ball-Valve Prostheses in the Mitral Valve Position.

Previous Serial Number: None

Principal Investigator: Stanton P. Nolan, M. D.

Other Investigators: David Gilbert, M. D.
Scott Stewart, M. D.
Thomas J. Fogarty, M. D.
Andrew G. Morrow, M. D.

Cooperating Unit: Division of Computer Research and Technology

Man Years
Total: 3
Professional: 2
Other: 1

Project Description: The disc valve and the ball valve are the two most widely used mitral valve prostheses. In order to determine which of these valves should provide the best clinical results, the mitral valves of four calves were replaced with a ball valve, and in six calves with a disc valve. Each prosthesis incorporated a flow probe in its base. Intracardiac pressures and flows were measured at different heart rates and cardiac outputs. The data were recorded on magnetic tape and converted from analog to digital form. A high speed digital computor was used to analyze the data and provided the following information: 1] transvalvular energy loss; 2] transvalvular power loss; 3] valvular input work; and, 4] valvular efficiency.

A statistical comparison of the performance of the two types of valves is being performed to determine which has the best operating characteristics.

Proposed Course: Completion of project and publication.

Serial No. -NHI-315
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Quantification of Aortic Insufficiency

Previous Serial Number: None

Principal Investigator: Stanton P. Nolan, M. D.

Other Investigators: R. Darryl Fisher, M. D.
Andrew G. Morrow, M. D.

Man Years
Total: 3/4
Professional: ½
Other: ¼

Project Description: To date there has been no practical method for the precise quantification of the percent of aortic insufficiency (AI) at the time of cardiac catheterization. A new catheter tip velocitometer was applied to the assessment of AI in dogs, and the results were compared to measurements made using standard techniques of electromagnetic flowmetry.

Four dogs with normal aortic valves and four dogs with chronic AI were studied. The results indicated a high degree of correlation (r=0.96) between the standard flow measurement and the measurements made with the catheter tip velocitometer in dogs with normal aortic valves. In those animals with aortic insufficiency the following correlation coefficients (r) and percent error (S) were found: 1) Percent regurgitation r=0.91, S= ±15%; 2) Forward stroke volume r=0.95, S= ±12%; 3) Regurgitant volume r=0.91, S= ±22%. It was concluded that the assessment of aortic insufficiency could be performed reliably with a catheter tip velocitometer.

Proposed Course. Completion of manuscript and publication.

Serial No. NHI-316
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Instantaneous Blood Flow Across the Normal Mitral Valve

Previous Serial Number: None

Principal Investigator: Stanton P. Nolan, M. D.

Other Investigators: Sewell H. Dixon, M. D.
R. Darryl Fisher, M. D.
Andrew G. Morrow, M. D.

Man Years

Total:	4
Professional:	2½
Other:	1½

Project Description: The blood flow across the mitral valve determines ventricular filling and, therefore, ventricular performance. An electromagnetic flow probe was sutured within the left atrium just above the mitral valve in 11 calves and measurements made of intracardiac flows and pressures.

Cardiac output, heart rate, and cardiac rhythm were varied. In some animals insufficiency of the mitral and/or aortic valves was produced. These measurements were analyzed in terms of the factors that influence ventricular filling under normal and pathological conditions.

The results indicated that 1) a properly timed atrial contraction may augment ventricular filling by 20%; 2) mass acceleration effects allow forward flow through the mitral valve to continue for 10-15 msec. after reversal of the A-V pressure gradient; 3) the normal valve closes without regurgitation; 4) a premature ventricular contraction may cause regurgitation of less than 5% of the stroke volume. It was concluded that: 1) atrial contraction is not required for nonregurgitant closure of the valve. 2) The major role of atrial contraction is the augmentation of ventricular filling.

Proposed Course: Completion and publication.

Serial No. -NHI-317
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Instantaneous Blood Flow Across a Mitral Ball Valve Prosthesis: Effects of Heart Rate and Cardiac Output on Transvalvular Energy Loss

Previous Serial Number: None

Principal Investigator: Stanton P. Nolan, M. D.

Other Investigators: Scott Stewart, M. D.
Thomas J. Fogarty, M. D.
Sewell Dixon, Jr., M. D.
Andrew G. Morrow, M. D.

Man Years

Total:	2
Professional:	1
Other:	1

Project Description: Prostheses are widely employed for the replacement of diseased cardiac valves. These prostheses do not duplicate the normal valve in their physical characteristics. Information concerning the hemodynamic behavior of these valves is needed in order to determine the optimal operating conditions and detrimental effects.

The mitral valves of calves were replaced with ball valve prostheses which incorporated an electromagnetic flow probe. Measurements were made of: 1] EKG; 2] Aortic, left ventricular, and left atrial pressures; 3] Aortic and mitral valve flow. From these measurements the power and energy losses across the valve were calculated and compared at different heart rates and levels of cardiac output.

Results indicated that optimal performance of a ball valve in the mitral position occurs at heart rates between 80 and 100 per minute and with cardiac outputs up to 3000 cc. per minute. At a constant cardiac output transvalvular power loss and energy loss increase if heart rate is increased. At a constant heart rate energy loss increases disproportionately above a critical level of cardiac output. From these studies it was concluded that the ball valve prosthesis in the mitral position may have adverse effects on cardiac performance unless cardiac output and heart rate are maintained within a narrow range.

Proposed Course: Project completed and manuscript submitted for publication.

Serial No.-NHI-318
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Pulmonary Vein Flow in Normal and Pathological States

Previous Serial Number: None

Principal Investigator: Sewell H. Dixon, Jr., M. D.

Other Investigators: Stanton P. Nolan, M. D.
Andrew G. Morrow, M. D.

Man Years

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

Project Description: The pulmonary veins can be considered a reservoir for left atrial filling. However, disagreement exists concerning the major determinants of pulmonary vein flow (PVF). The vis-a-tergo effect of the right ventricle is considered most important by some authors, whereas others emphasize the vis-a-fronte of the left atrium. Additionally, no studies are available concerning pathological alterations in PVF. Measurements of PVF have been obtained in dogs using an electromagnetic cannulating flow probe with simultaneous registration of the EKG and systemic, left atrial, and left ventricular pressures. The reproducibility of the method has been demonstrated in open and closed chest preparations. PVF is forward, toward the atrium, throughout the cardiac cycle. Rapid acceleration begins with opening of the mitral valve; deceleration occurs early in ventricular systole. A small secondary increase in flow is observed prior to the atrial "v" wave with a rapid decline immediately before the "y" descent. Peak flow of 800-1000 cc./min. and mean flows of 400-500 cc./min. have been recorded.

Reversal of PVF has been observed, throughout the cardiac cycle, with pulmonary artery occlusion or bronchial obstruction. Mitral insufficiency or premature ventricular contractions produce systolic reversal of flow. Further studies concerning the effects of alterations in left atrial pressure, changes in heart rate, and various arrhythmias upon pulmonary vein flow are in progress.

Proposed Course: Completion of project and publication.

Serial No. -NHI-319
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A Catheter Technique for the Removal of Pulmonary Emboli Without Cardiopulmonary Bypass

Previous Serial Number: None

Principal Investigator: R. Darryl Fisher, M. D.

Other Investigators: Stanton P. Nolan, M. D.
Thomas J. Fogarty, M. D.

Man Years
Total: 1
Professional: ½
Others: ½

Project Description: Venous thrombosis resulting in fatal pulmonary embolism remains one of the most serious and dreaded complications in clinical medicine. Surgical removal of pulmonary emboli (PE) requires a major operative procedure with temporary interruption of circulation. This study was undertaken to evaluate the feasibility of removal of PE through a specially designed, Teflon-reinforced silastic catheter (5 mm. I.D.).

Twelve dogs were subjected to PE by infusion of homologous blood clot. Approximately 1.5-2.0 cm./Kg. of 7 mm. clot was injected in each animal. Pulmonary angiograms were made before and after removal of PE. In 5 dogs, the catheter was inserted via the vena cava at thoracotomy, and manipulated into the pulmonary artery (PA) and its branches. Gentle suctioning and flushing through the catheter was successful in removing all PE in 4 of 5 dogs; in the other dog small amount of lower lobe clot remained. In the other 7 dogs without thoracotomy, a cardiac catheter (8F) was directed under fluoroscopic control into the PA, and the larger embolectomy catheter was then passed over the guide catheter into each PA and its branches. In each of 7 dogs gentle flushing and suctioning was successful in removing all of the PE except for a small right lower lobe clot in 6 animals. Although of large size, the presence of the embolectomy catheter in the PA did not cause significant changes in PA or right ventricular pressure in control animals without PE.

Proposed Course: The preliminary experimental study has been completed and the manuscript is in preparation. Future clinical use will depend upon additional fabrication changes and evaluation in the human cadaver.

Serial No. -NHI-320 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Postoperative Respiratory Managment of Open Heart Patients

Previous Serial Number: None

Principal Investigator: Lee P. Enright, M. D.

Other Investigators: Hamner Hannah, M. D.
Eric Johnson, M. D.
Andrew G. Morrow, M. D.

Man Years
Total: 1½
Professional: 1½
Other: 0

Project Description: At the National Heart Institute, the respiratory care of postoperative open heart patients usually takes one of two forms: either positive pressure respiration is given via the endotracheal tube for the first 16 - 24 hours postoperatively, or the endotracheal tube is removed when the patient is fully awake.

The charts of 200 patients operated on since July, 1966 were reviewed. This group was made up of two subgroups of 100 consecutive patients managed by each of the two modes of therapy. Each patient chart was reviewed for age, sex, lesion, history of smoking, history and physical findings of respiratory tract disease, catheterization findings and history of previous surgery. The intraoperative course was described as to operation, length of anesthesia, length of cardiopulmonary bypass and anesthetic agent. The postoperative blood gases and the incidence of postoperative tracheostomy, pneumonitis, atelectasis, arrhythmia, and psychosis were tabulated.

The two groups of patients will be presented in two ways. First, descriptive detail of the two groups will give an operational description of the present indications here for management by a particular method. Secondly, inter-group overlaps in terms of preoperative and intraoperative descriptive information will be analyzed to assess the relative morbidity of the two methods of management.

The data has been collected from the charts and is presently undergoing analysis.

Proposed Course: Completion and publication.

Serial No. -NHI-321
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Evaluation of Methods for Protecting the Myocardium During Aortic Cross Clamping.

Previous Serial Number: None

Principal Investigators: Lee P. Enright, M. D.
Robert L. Reis, M. D.

Other Investigators: Rudolf Staroscik, M. D.
Andrew G. Morrow, M. D.

Man Years
Total: 1
Professional: ½
Other: ½

Project Description: Anoxic cardiac arrest, direct coronary artery perfusion and regional or total body hypothermia have been employed during open heart operations when aortic cross clamping is necessary. The effects of these methods of protecting the myocardium during periods of interruption of normal coronary arterial flow on subsequent cardiac performance are being assessed in dogs. Force-velocity curves, length-tension curves and diastolic pressure-volume relationships were determined before and after 30, 45 and 60 minutes of aortic cross clamping at 37°C. Return of near normal function occurred after 30 minutes, but significant and sustained injury resulted after 45 or 60 minutes of aortic cross clamping. Coronary artery perfusion at different flow rates and temperatures will be assessed as well as regional cardiac hypothermia induced without coronary perfusion.

Proposed Course: Completion of study and preparation of a manuscript for publication.

67

Serial No. NHI-322
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effects of Epicardiectomy on the Performance of the Acutely Ischemic Left Ventricle.

Previous Serial Number: None

Principal Investigator: Robert L. Reis, M. D

Other Investigators: Lee P. Enright, M. D.
Hamner Hannah III, M. D.
Andrew G. Morrow, M. D.

Man Years
Total: 1 1/3
Professional: 2/3
Other: 2/3

Project Description: The effects of epicardiectomy on left ventricular function were assessed in dogs in which the left anterior descending coronary artery was temporarily occluded. Epicardiectomy significantly improved the performance of the acutely ischemic left ventricle as determined by ventricular function curves, length-tension curves, and force velocity relations. A significant increase in dp/dt max. and a lessened tendency for ventricular fibrillation also followed epicardiectomy. Diastolic left ventricular compliance was not altered. Improved ventricular performance was observed when there was no opportunity for blood to enter the myocardium from the left ventricular lumen, suggesting that this mechanism is not responsible for the functional improvement which occurred. It has been demonstrated that cardiac denervation decreases ventricular irritability following coronary artery ligation. Whether or not the improvement in ventricular performance which occurred following epicardiectomy is also related, in some way, to neural ablation requires further study.

Proposed Course: Project completed and submitted for publication to the Journal of Thoracic and Cardiovascular Surgery.

-268

Serial No. -NHI-323
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Left Ventricular Function After Formation of the Myocardial Tunnels Used for Internal Mammary Artery Implantation.

Previous Serial Number: None

Principal Investigators: Lee P. Enright, M. D.
Robert L. Reis, M. D.

Other Investigators: Hamner Hannah III, M. D.
Andrew G. Morrow, M. D.

Man Years
Total: 1
Professional: ½
Other: ½

Project Description: Left ventricular function curves were inscribed and dp/dt max. measured at constant heart rate and aortic pressure in 16 dogs before and after the formation of myocardial tunnels used for internal mammary artery implantation. In 5 dogs (Group I) a small epicardial incision was made and a long myocardial tunnel approximately midway between endocardium and epicardium was developed by blunt dissection extending for a distance approximately one-half the ventricular length. Following the formation of an anterior and posterior tunnel the freely bleeding right and left internal mammary arteries were implanted. In 5 dogs (Group II) tunnels of similar size and location were created by sharp dissection with a scalpel blade. In 6 additional dogs (Group III) myocardial infarction and impaired left ventricular function were produced by left anterior descending artery ligation 6 weeks prior to the time of study. In these animals tunnels of similar size and location were created by blunt dissection and after the inscription of function curves the tunnels were enlarged by sharp dissection with a scalpel following which a repeat function curve was performed. In 15 animals the ventricular function curve inscribed after tunnel formation was similar to the control curve and dp/dt max. was also unchanged. In one dog from Group II moderate depression of ventricular function followed tunnel formation and was undoubtedly related to laceration of a large coronary artery branch.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

These data indicate that neither the creation of large myocardial tunnels nor the implantation of the freely bleeding internal mammary arteries produce significant immediate alteration in the function of the normal or functionally impaired myocardium. The increased operative mortality associated with the simultaneous implantation of both mammary arteries compared to single artery implantation is probably related to factors other than injury produced by myocardial tunnel formation.

Proposed Course: Study is completed and is being prepared for publication.

Serial No. -NHI-324 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Anomalous Origin of the Left Coronary Artery from the Pulmonary Artery: Hemodynamic Assessments and the Pattern of Instantaneous Flow After Anastomosis to the Aorta.

Previous Serial Number: None

Principal Investigators: Robert L. Reis, M. D.

Other Investigators: Lawrence S. Cohen, M. D.
Dean T. Mason, M. D.
Andrew G. Morrow, M. D.

Man Years

Total:	1/12
Professional:	1/12
Other:	0

Project Description: An anomalous left coronary artery was excised from the pulmonary artery and connected by a reversed segment of autogenous saphenous vein to the ascending aorta in a 20 year old boy. Before it was anastomosed to the aorta, retrograde flow of oxygenated blood from coronary artery to pulmonary artery occurred predominantly during diastole; the pressure in the main left coronary artery was 20/9 mm. Hg and rose to systemic levels following occlusion of the coronary artery at its origin. After transplantation the vessel was encircled with an electromagnetic flow probe, and instantaneous left coronary artery flow was measured simultaneously with left ventricular and aortic pressure. With the onset of diastole there was rapid acceleration of coronary flow followed by a gradual decrease as aortic diastolic pressure fell. During isometric ventricular contraction coronary blood flow decreased rapidly, and at peak isometric tension forward flow ceased for 0.01 sec. Approximately 70% of total left coronary flow occurred during ventricular diastole and 30% during ventricular systole. Mean flow through the left coronary artery was 100 cc./min. These are apparently the first observations of instantaneous coronary flow in man.

One month postoperatively the left ventricular end-diastolic pressure had decreased to 10. mm. Hg from an abnormal preoperative level of 25 mm. Hg, and cineangiography demonstrated patency of the anastomosis with filling of the entire left coronary system.

Proposed Course: A manuscript is being prepared for publication.

Serial No. -NHI-325
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Description: The Effect of Excision of the Sino Atrial Node on Cardiac Rate and Rhythm.

Previous Serial Number: None

Principal Investigator: Robert L. Reis, M. D.

Other Investigators: Hanmer Hannah III, M. D.
Lee P. Enright, M. D.
William C. Roberts, M. D.
Andrew G. Morrow, M. D.

Man Years
Total: ½
Professional: ¼
Other: ¼

Project Description: The sino atrial node was excised in 15 mongrel dogs after control heart rate and rhythm were recorded. Immediately following removal of the sino atrial node, A-V nodal rhythm occurred and the heart rate decreased approximately 20 - 30%, however, after 2 - 7 months sinus rhythm recurred at heart rates comparable to the control valves. Histologic examination of the tissue removed at the time of operation is being performed to confirm complete removal of the sino atrial node.

These data suggest that sinus rhythm may be present after excision of the sino atrial node.

Proposed Course: Histologic evaluation of the tissue is being performed following which a manscript will be prepared for publication.

872

Serial No. -NHI-326
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effect of Epicardiectomy on Left Ventricular Function and Diastolic Compliance

Previous Serial Number: None

Principal Investigators: Hamner Hannah III, M. D.
Robert L. Reis, M. D.

Other Investigators: Lee P. Enright, M. D.
Andrew G. Morrow, M. D.

Man Years
Total: 1
Professional: ½
Other: ½

Project Description: Left ventricular function curves were inscribed and dp/dt max. measured at fixed heart rate and constant aortic pressure before and after epicardiectomy in 15 dogs. In 4 additional dogs isovolumetric ventricular contractions were obtained by inserting a latex balloon attached to a metal cannula into the cavity of the left ventricle through a stab incision at its apex during cardiopulmonary bypass. Ventricular volume was established by inflating the balloon with a measured volume of saline. The mitral valve orifice was occluded with a specially fabricated plug, and drains placed to recover blood returned to the left ventricle through thebesian veins. At constant aortic pressure and fixed heart rate this preparation provided data from which length-tension curves (peak systolic tension versus ventricular volume) were inscribed, the maximum velocity of shortening of ventricular contractile elements determined, and ventricular diastolic compliance assessed (end-diastolic pressure versus ventricular volume curves), before and after epicardiectomy.

Ventricular function curves, left ventricular dp/dt max., length-tension curves, and left ventricular diastolic compliance curves were similar before and after epicardiectomy in all animals. The maximum velocity of shortening of the ventricular contractile element averaged 0.73 cm./sec./cm. ventricular circumference before and 0.79 cm./sec./cm. ventricular circumference after epicardiectomy.

These data indicate that epicardiectomy does not impair the function or alter the diastolic compliance of the normal canine left ventricle.

Proposed Course: Study is completed and is being prepared for publication.

'73

Serial No. -NHI-327
1. Clinic of Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effects of Acute Coronary Artery Occlusion in Animals With Functioning Internal Mammary Artery Implants.

Previous Serial Number: None

Principal Investigator: Robert L. Reis, M. D.

Other Investigators: Lee P. Enright, M. D.
Hamner Hannah, III, M. D.
Andrew G. Morrow, M. D.

Man Years

Total:	1
Professional:	½
Other:	½

Project Description: Operations designed to revascularize the ischemic myocardium have been receiving increasing consideration and clinical application. This renewed interest was stimulated by the cineangiographic demonstration in patients of the validity of Vineberg's concept that the internal mammary artery, when implanted into ventricular myocardium, would remain patent. There is little evidence, however, which indicates that the presence of a functioning implant reduces mortality or morbidity following acute coronary artery occlusion.

Thirty dogs compromised 2 groups. In 15 dogs an ameroid occluder was placed about the origin of the left circumflex coronary artery and a Chocker placed around the origin of the left anterior descending vessel. The Chocker was buried in the subcutaneous tissues and could be retrieved at a later date and the anterior descending artery occluded while the dog was awake. Both the right and left internal mammary vessels were then implanted into the left ventricular myocardium. In 15 additionals dogs an identical procedure was performed, but the mammary vessels were not implanted.

Nine months following operation the anterior descending coronary artery will be occluded while the animal is awake and the electrocardiogram is being recorded. The hearts of all animals will be examined after death or after sacrifice and examined pathologically.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

It is hoped that this study will provide information regarding the benefit, if any, provided by functioning internal mammary implants to animals in which acute coronary artery occlusion is produced.

Proposed Course: Completion of study and preparation of a manuscript for publication.

Serial No. -NHI-328
1. Clinic of Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A New Method for the Maintenance of Induced Ventricular Fibrillation During Cardiac Operations.

Previous Serial Number: NHI-107

Principal Investigator: Robert L. Reis, M. D.

Other Investigators: Andrew G. Morrow, M. D.
Michael Griefner

Cooperating Units: Biomedical Engineering Branch

Man Years
Total: 3/4
Professional: 3/4
Other: 0

Project Description: Previous studies in our laboratory have demonstrated that elective ventricular fibrillation, maintained by the constant application of an a. c. current to the ventricle seriously impairs subsequent cardiac performance. A more satisfactory method of dependably maintaining ventricular fibrillation was developed in order that induced ventricular fibrillation might be employed safely during open cardiac operations. This new method was evaluated in animals and recently has been employed in the operating room on patients in whom elective ventricular fibrillation is needed. Continuing clinical experience (14 pts.) has resulted in some modifications of the stimulator and electrodes used.

Proposed Course: Further clinical trial and eventual routine use of this technique for induced ventricular fibrillation.

Serial No. -NHI-329 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Anatomic Studies of Hearts Containing Caged-ball Prosthetic Valves.

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigator: Andrew G. Morrow, M.D.

Man Years

Total:	1/6
Professional:	1/12
Other:	1/12

Project Description: An analysis of the necropsy observations was made in 98 patients who had previously undergone replacement of one or more cardiac valves with Starr-Edwards Prostheses. Sixty-five had died within one month of operation and 33 at later periods up to 56 months postoperatively. The initial technical problems involved in the insertion have, for the most part, been eliminated, and the immediate operative mortality now is low. What period of survival may be expected for these individuals, however, remains in doubt. Prosthetic thrombosis continues to be a major problem which has been altered little by anticoagulant therapy. Regurgitation around prosthetic valves is becoming less frequent as more experience is being gained in their insertion. Degeneration of the aortic prosthetic ball is becoming a major problem. If the ball swells, its movement becomes limited; if the ball becomes smaller, it may embolize. The use of a metal ball may lessen the problem of ball variance, and the covering of the metallic struts may lessen the tendency for thrombus to develop on them. Infection at the site of the prostheses remains an ever dangerous problem, particularly since a precipitating cause for the infection is often not apparent. The cause of sudden death, usually following aortic valve replacement, continues to be the most frequent mode of exitus, and only occasionally is an anatomic explanation apparent.

Thus, cardiac valve replacement had added a new and temporarily effective therapy for patients with severe valvular cardiac disease. Valve replacement at this time, however, should be reserved for the patient who is severely limited (functional class III or IV) by his valvular disease.

Publications: Roberts, W. C. and Morrow, A. G.: Anatomic studies of hearts containing caged-ball prosthetic valves. Johns Hopkins Med. J. 121:271, October 1967.

Serial No. -NHI-330 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Fatal Degeneration of the Silicone Rubber Ball of the Starr-Edwards Prosthetic Aortic Valve.

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigator: Andrew G. Morrow, M.D.

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: Among 46 patients who died one month or later following replacement of one or more cardiac valves with Starr-Edwards prostheses, 11 died because of severe degeneration of the silicone rubber aortic ball. Thirty-five of the 46 patients had undergone aortic valve replacement, and 11 isolated mitral valve replacement. The interval between operation and death in each of the 11 patients was 24 months or over. One additional patient, who had died longer than 24 months following aortic valve replacement, also showed evidence of aortic ball degeneration, but it was not severe enough to cause clinical disturbance. In none of the 11 patients who died late after isolated mitral replacement had evidence of degeneration of the silicone rubber ball. Likewise, none of the 23 autopsied patients who died less than 24 months after aortic valve replacement had evidence of aortic ball variation. In 11 of the 12 patients with aortic ball degeneration, 6 had atrophied, fractured, and dislodged aortic balls and 5 had balls which had swollen and become impacted within the prosthetic cage. Eight of the 11 patients with severe evidence of ball degeneration died suddenly and in the other 3 had congestive heart failure for some period before they died.

This study suggests that ball degeneration after replacement of the aortic valve with a silicone rubber ball may be an invariable late consequence of replacement of the aortic ball with this type prosthesis. It is hoped that the use of a metal ball will prevent this complication.

Proposed Course: This study has been accepted for publication in the American Journal of Cardiology.

Serial No. -NHI-331 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Secondary Left Ventricular Endocardial Fibroelastosis Following Mitral Valve Replacement. Cause of Cardiac Failure in the Late Postoperative Period.

Previous Serial Number: None

Principal Investigator: William C. Roberts, M. D.

Other Investigator: Andrew G. Morrow, M. D.

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: In studying the hearts of patients who died following replacement of one or more cardiac valves with prostheses, it was observed that endocardial fibrosis occurs not infrequently in the left ventricle following mitral valve replacement. Thus, in a systematic fashion the hearts of all patients who died following valve replacement were examined for the absence or presence of left ventricular endocardial fibrosis. This finding was not observed in any patient who died within a month of operation and was not found in any patients who died after isolated aortic valve replacement. In contrast, of 16 patients who died in the late postoperative period following mitral replacement, extensive endocardial fibrosis was found in 14 of them. In 2 of these 14 patients it was convincing at necropsy that death in them was a result of the endocardial fibrosis which was not associated with coronary arterial narrowing. The cause of endocardial thickening was believed to be the result of turbulence produced within the left ventricular cavity by the caged-ball Starr-Edwards mitral prosthesis.

Publications: Roberts, W. C. and Morrow, A. G.: Secondary left ventricular endocardial fibroelastosis following mitral valve replacement: Cause of cardiac failure in the late postoperative period. Circulation Supp. II-37:101, April, 1968.

Serial No. -NHI-332 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Calcific Pulmonic Stenosis

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: Dean T. Mason, M.D.
Andrew G. Morrow, M.D.
Eugene Braunwald, M.D.

Cooperating Unit: Cardiology Branch, NHI

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: Seven patients are described in whom calcium deposits occurred in stenotic pulmonic valves. In 5 patients the calcific deposits were visible on chest roentgenograms. Survival into adulthood and severe pulmonic stenosis appeared to be the prerequisites for the development of large deposits of calcium in this valve. This study was prompted by the fact that calcific stenosis of the aortic valve is a well recognized and relatively common clinical and pathologic entity. In contrast, calcific pulmonic stenosis is a rare lesion.

Proposed Course: This work has been accepted for publication in Circulation.

Serial No. -NHI-333 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Chronic Mitral Regurgitation of Unresolved Etiology in the Elderly: A Clinico-pathologic Study of Two Patients.

Previous Serial Number: None

Principal Investigator: William C. Roberts, M. D.

Other Investigators: Richard S. Ross, M. D.
Joseph C. Eggleston, M. D.
Rashid A. Massumi, M. D.

Cooperating Units: Departments of Medicine, Radiology and Pathology, The Johns Hopkins University School of Medicine, and the Cardiopulmonary Laboratory, D. C. General Hospital.

Man Years

Total:	1/6
Professional:	1/12
Other:	1/12

Project Description: In previous studies from this clinic, pure mitral regurgitation was found to be the result of rheumatic heart disease in approximately 50% of patients and to be of nonrheumatic etiology in the other 50%. Of the latter group, the most common cause of mitral regurgitation was found to be infective endocarditis with rupture of chordae tendineae. Other causes included congenital malformations, the Marfan's syndrome, and various conditions affecting the papillary muscles and the size of the left ventricular cavity. Of a large group of patients with pure mitral regurgitation, 2 were studied in whom the cause was not discernible despite extensive clinical and necropsy studies. Each of these 2 patients were old, ages 60 and 84 years, respectively. At necropsy the mitral leaflets and chordae tendineae in each were found to be virtually normal. In one patient the papillary muscles in left ventricular walls were entirely normal, but in the second patient one of the papillary muscles and the wall of the left ventricle beneath it was extensively scarred. The cause of this focal scarring, however, was not known since the coronary arteries were widely patent. It was speculated that the papillary muscle scarring in this latter patient resulted from a viral myocardial infection many decades earlier.

Publication:

Roberts, W. C., Ross, R. S., Eggleston, J. C., and Massumi, R. A.: Chronic mitral regurgitation of unresolved etiology in the elderly: A clinico-pathologic study of two patients. JOHNS HOPKINS MED. J. 122: 26-36, January 1968.

Serial No. -NHI-334 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Isolated Acquired Tricuspid Regurgitation. With a Note on Renal Lesions on Isolated Right-Sided Endocarditis.

Previous Serial Number: None

Principal Investigator: D. Luke Glancy, M.D.

Other Investigators: Frank I. Marcus, M.D.
Michael Cuadra, M.D.
Gordon A. Ewy, M.D.
William C. Roberts, M.D.

Cooperating Unit: Georgetown University Medical Division, D. C. General Hospital and the Department of Pathology, D.C. General Hospital.

Man Years

Total:	1/6
Professional:	1/12
Other:	1/12

Project Description: Two patients, each heroine addicts, died from tricuspid regurgitation which was secondary to infective endocarditis on a previously normal valve. This study proposed to emphasize that acquired isolated tricuspid regurgitation is due almost entirely to infective endocarditis and that most of these patients, as were the 2 studied, were heroine addicts. Although glomerulonephritis is fairly common in patients with left-sided infective endocarditis, it is a rare complication of isolated right-sided endocarditis. In one of the patients studied, however, glomerulonephritis not only occurred but proved to be fatal. The histologic features of this fatal glomerulonephritis were studied extensively. Attempts to learn why infective endocarditis in heroine addicts occurs so frequently on the right-sided cardiac valves rather than on the left-sided ones was not elucidated by this study.

Proposed Course: This work is in press in the American Journal of Cardiology.

Serial No.-NHI-335 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Cardiac Valvular Lesions in Rheumatoid Arthritis.

Previous Serial Number: None

Principal Investigator: William C. Roberts, M. D.

Other Investigators: James A. Kehoe, M. D.
Deborah F. Carpenter, M. D.
Abner Golden, M. D.

Cooperating Units: Departments of Pathology and Medicine
Georgetown University Medical Center

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: Rheumatoid nodules have been observed in the hearts of patients with rheumatoid arthritis for sometime. Detailed descriptions of the location of these nodules in cardiac valves, however, has not been done. Two patients were studied at autopsy each of whom had severe rheumatoid arthritis and in each characteristic rheumatoid nodules were found in each of the 4 cardiac valves. The specific locations of the nodules in the valves were found to be characteristic of this entity. The nodules were found to occur within the substance of the valve and the peripheral portions of the valve leaflets were uninvolved. The type of cardiac valvular disease observed in patients with rheumatoid arthritis is highly specific and differs strikingly morphologically from the valve lesions observed in patients with carcinoid heart disease, rheumatic heart disease, congenital heart disease, and the valvular disease seen in the Marfan's syndrome, Hurler syndrome and in the pulseless disease syndrome.

Proposed Course: This study was accepted for publication in the Archives of Internal Medicine.

Serial No.-NHI-336 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS - NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Papillary Muscle Fibrosis in Primary Myocardial Disease.

Previous Serial Number: None

Principal Investigator: Frank I. Marcus, M.D.

Other Investigators: Lucia Gomez, M.D.
D. Luke Glancy, M.D.
Gordon A. Ewy, M.D.
William C. Roberts, M.D.

Cooperating Unit: Georgetown University Medical Division, D. C. General Hospital & Washington Hospital Center.

Man Years

Total:	1/6
Professional:	1/12
Other:	1/12

Project Description: Since disease of the papillary muscles was first emphasized in 1960, fibrosis of them have been described in a number of diseases. The present study showed that papillary muscle fibrosis may occur in patients with primary myocardial disease who have entirely normal coronary arteries. The patient studied was 39 years old and she had clinical evidence of mitral regurgitation for 3 years. At necropsy the mitral leaflets and chordae tendineae were entirely normal, but one of the papillary muscles was severely scarred. Thus, this study showed that coronary arterial narrowing is not the only cause of scarring of left ventricular papillary muscles.

Proposed Course: This paper was accepted for publication in the American Heart Journal.

Serial No. -NHI-337 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Muscular Subaortic Stenosis in an Elderly Patient: Clinical and Pathological Observations.

Previous Serial Number: None

Principal Investigator: Gordon A. Ewy, M.D.

Other Investigators: Frank I. Marcus, M.D.
Oshin Bohjalian, M.D.
Henry Burke, M.D.
William C. Roberts, M.D.

Cooperating Unit: Georgetown University Medical Division, Department of Pathology, D. C. General Hospital

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: Since idiopathic hypertrophic subaortic stenosis was described in 1958 a number of reports have emphasized various features of this entity. The majority of patients with IHSS have been in the younger age group and this study simply called attention to the fact that this disease may also occur in the elderly. The patient studied was a 74-year-old woman who had systemic hypertension in the past and who had all of the classic clinical features of IHSS.

Proposed Course: This paper was accepted for publication in the American Journal of Cardiology.

Serial No. -NHI-338
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Malformations of the Aortic Valve in Patients with Tetralogy of Fallot.

Previous Serial Number: None

Principal Investigator: D. Luke Glancy, M.D.

Other Investigators: Andrew G. Morrow, M.D.
William C. Roberts, M.D.

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: Although right-sided aortic arches, bicuspid or absent pulmonic valves, atrial septal defects, persistent left superior venae cavae and pulmonary arterial branch stenoses have been recognized as malformations which frequently occur in patients with tetralogy of Fallot, abnormalities of the aortic valve have been described in these patients very rarely. The finding of aortic regurgitation in a patient with right ventricular outflow obstruction and ventricular septal defect stimulated us to examine the aortic valves of other patients with tetralogy of Fallot studied by us at necropsy. The hearts of 45 patients were examined and 5 were found to have anatomic malformations of the aortic valve. Four of the 5 were adults. In 2 abnormalities on physical and roentgenographic examination disclosed hemodynamic dysfunction of the aortic valve and death in one of them was related to stenosis of the aortic valve. In a third patient, a small gradient was found at catheterization across the aortic valve, but in the other 2 patients no hemodynamic dysfunction of this valve was observed.

Thus, anatomic malformations of the aortic valve may occur in slightly over 10% of patients with tetralogy of Fallot, and may cause functional disturbance in about half of those involved. The purpose of this report was simply to call attention to the occurrence of abnormalities of the aortic valve in patients with Fallot's tetralogy.

Proposed Course: This study was accepted for publication in the American Heart Journal.

Serial No. NHI-339 (c)
1. Clinic of Surgery
2. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Aneurysmal Dilatation of the Coronary Arteries in Cyanotic Congenital Cardiac Disease: Report of a 40 -year-old Patient with Taussig-Bing Complex.

Previous Serial Number: None

Principal Investigator: Joseph K. Perloff, M. D.

Other Investigators: Charles W. Urschell, M. D.
William C. Roberts, M. D.
Walter H. Caulfield, M. D.

Cooperating Unit: Depts. of Medicine and Pathology
Georgetown University School of Medicine and the Division of Cardiology, Georgetown University Hospital

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: Marked dilatation and tortuosity of the coronary arteries was observed in a 40-year-old woman with the Taussig-Bing complex who was known to be severely cyanotic all her life. It was speculated that the aneurysmal dilatation resulted from compensatory dilatation of these arteries because of the severe desaturation of the arterial blood in this patient. In a normal person the oxygen in the coronary arteries is nearly 100% saturated. Therefore, the only means of increasing myocardial nutrition in a patient with severe arterial desaturation is by increasing the coronary arterial flow. This is possible by increasing the size of the cornary arteries which occurred in the present patient. The other unusual feature in the present patient was the fact that she lived for 40 years with the condition in which the aorta arises from the right ventricle and the pulmonary trunk arises from both ventricles. It is truly amazing that she lived this long since there was no obstruction in the pulmonary arteries in her lungs. Indeed, the pulmonary arteries were normal. This patient was the first to be described in whom the coronary arteries were observed to be aneurysmally dilated in cyanotic congenital heart disease.

Proposed Course: Accepted by the American Journal of Medicine.

Serial No.-NHI-340 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Anomalous Left Ventricular Band: An Unemphasized Cause of a Precordial Musical Murmur.

Previous Serial Number: None

Principal Investigator: William C. Roberts, M. D.

Other Investigators: None

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: Conditions which temporarily increase the speed of blood flow through the heart or great vessels are usually the cause of a transient precordial murmur. These states which have been responsible for a so-called functional or innocent murmur are nervousness, anemia, fever, tachycardia, exercise, hyperthroidism and pregnancy. Transient dilatation of the cardiac ventricles from any cause resulting in stretching of the atrioventricular rings and papillary muscles may be responsible for a precordial systolic murmur which also may subsequently disappear. Likewise, transient papillary muscle dysfunction is recognized as a cause of a transient as well as a permanent precordial systolic murmur. The present study attempts to describe another but completely unemphasized cause of a transient precordial murmur and that is an anomalous band in the left ventricle. The patient described was a 68-year-old man who on clinical examination was confirmed to have a systolic murmur which subsequently disappeared. Initially when the murmur was present, the patient was in severe heart failure and when the excessive fluid disappeared the murmur disappeared. The patient suddenly died and at necropsy an anomalous band was found to be present within the left ventricular cavity extending from the ventricular septum to the lateral free wall of the left ventricle. Other hearts were examined in which ventricular bands were also seen, but clinical evidence of a murmur in them was not apparent. The report emphasizes that the anomalous bands are of no importance other than their being confused with a more serious condition.

Proposed Course: The paper has been accepted for publication in The American Journal of Cardiology.

Serial No.-NHI-341 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Rocks in the Right Ventricle: A Complication of Congenital Right Ventricular Infundibular Obstruction Associated with Chronic Pulmonary Parenchymal Disease.

Previous Serial Number: None

Principal Investigator: David C. Dean, M.D.

Other Investigators: Thomas Pamukcoglu, M.D.
William C. Roberts, M.D.

Cooperating Unit: Departments of Medicine and Pathology, State University of New York at Buffalo, The Veterans Administration Hospital, Buffalo, New York.

Man Years

Total:	1/6
Professional:	1/12
Other:	1/12

Project Description: Calcium is frequently observed in the heart, but it occurs mainly in coronary arteries, in mitral or aortic leaflets or annulae and occasionally in left atrial or left ventricular mural thrombi, in thickened pericardia, occasionally in intracardiac tumors, and at times in myocardial fibers. A patient was studied in whom 11 isolated calcified stones were found in the right ventricular cavity. The patient was a 56-year-old man and during life had virtually no evidence of cardiac disease. At necropsy, he was found to have a mild degree of infundibular right ventricular outflow obstruction and 11 stones in the right ventricle. One of them was analyzed chemically and found to consist of 32% hydroxyl-apatite, 56% tricalcium phosphate, and 12% protein. The cause of these stones was not determined by pathologic examination, but similar patients were not found in reviewing the medical literature.

Proposed Course: This paper was accepted for publication in the American Journal of Cardiology.

Serial No.-NHI-342 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Endomyocardial Disease and Eosinophilia. A Clinical and Pathologic Spectrum.

Previous Serial Number: None

Principal Investigator: William C. Roberts, M. D.

Other Investigators: Donald G. Liegler, M. D.
Paul P. Carbone, M. D.

Cooperating Unit: Solid Tumor Service
Medicine Branch, NCI

Man Years

Total:	1/6
Professional:	1/12
Other:	1/12

Project Description: From 1953 until 1968, three patients were referred to the National Cancer Institute because of eosinophilic leukemia. Each of these patients had large numbers of mature eosinophils in the blood and severe, ultimately fatal, congestive cardiac failure. Necropsies disclosed extensive endomyocardial fibrosis with superimposed thrombi in each of the 2 patients that were autopsied. The observations in these 3 patients with "eosinophilic leukemia" were found to be virtually identical to those described in patients with Loffler's fibroplastic parietal endocarditis. The 3 patients described also had clinical courses similar to patients previously described with Loffler's endocarditis. For example, each patient was young (age 25 to 35 years), each had severe leukocytosis, blood eosinophilia, generalized cardiomegaly, intractable cardiac failure, renal dysfunction and a short illness with symptoms ranging from 11 to 26 months.

This study strongly suggests that eosinophilic leukemia is identical to what has been called in the past Loffler's fibroplastic parietal endocarditis. Furthermore, this study shows that Loffler's endocarditis and eosinophilic leukemia are similar to the African heart disease called endomyocardial fibrosis. The study suggests that endomyocardial fibrosis is simply an end stage of what initially may appear to be eosinophilic leukemia or fibroplastic parietal endocarditis. This study attempts to place under one disease heading at least 4 entities which previously had been considered 4 distinct diseases.

Proposed Course: This study was accepted for publication in The American Journal of Medicine.

Serial No. -NHI-343 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Fatal Acute Rheumatic Fever in Childhood Despite Corticosteroid Therapy. With a Note on the Spectrum of Childhood Rheumatic Fever.

Previous Serial Number: None

Principal Investigator: D. Luke Glancy, M.D.

Other Investigators: Rashid A. Massumi, M.D.
William C. Roberts, M.D.

Cooperating Unit: George Washington University Division of Medicine
D. C. General Hospital

Man Years

Total:	1/6
Professional:	1/12
Other:	1/12

Project Description: The incidence of death in childhood from rheumatic heart disease has been declining in the United States for 3 decades. The role of adrenocorticotropic hormone in the continuing decline in mortality during the past 2 decades has been debated widely and remains uncertain. Nevertheless, death from rheumatic carditis is now uncommon in children treated with these agents and is rare during an initial attack of rheumatic fever. Such occurred, however, in 2 children whom we studied. One patient, a 3-year-old child, died within 20 days from the onset of acute rheumatic fever. The second patient, a 9-year-old girl died 1 1/2 years after the onset of her illness. Death in her resulted from the mechanical lesion, mitral regurgitation, since histologic sections of myocardium were normal except for hypertrophied muscle fibers. In contrast, the 3-year-old child had numerous Aschoff bodies throughout the myocardium. Thus, acute rheumatic fever may, at times, be a fatal disease in childhood, but why it runs a fulminant course in one patient and produces a transient illness in another patient was not elucidated by this study.

Proposed Course: This work is in press in the American Heart Journal.

Serial No. -NHI-344(c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Prepulseless Phase of Pulseless Disease or Pulseless Disease with Pulses. A Newly Recognized Cause of Cardiac Disease, of Monoclonal Gammopathy, and of "Fever of Unknown Origin."

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: Rob Roy MacGregor, M.D.
G. David Beiser, M.D.
Sheldon M. Wolff, M.D.

Cooperating Units: Laboratory of Clinical Investigations, NIAID
Cardiology Branch, NHI

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: A patient was studied in the Clinical Center who was sick for a 14-month period and during this time, despite extensive clinical studies, a diagnosis was never made. The unusual clinical features were the presence of monoclonal gammopathy, atypical bacterium in the blood, mild mitral regurgitation and terminally heart failure. At necropsy, the 4 cardiac valves were found to be mildly but diffusely thickened, the aorta in its entirety was severely thickened, and all arteries arising from the aorta were narrowed. Histologic examination of the coronary arteries not only disclosed them to be occluded, but also to contain numerous inflammatory cells. Thus, this patient fell into the category of pulseless disease or Takayasu's arteritis. Previous reports on this entity have indicated nonspecific changes in the cardiac valves, but this study demonstrated that the changes in the cardiac valves appear to be specific for this entity and consists of diffuse fibrous thickening superimposed on the internal elastic membrane of the valve cusp. The valve cusps themselves remain normal and the superimposed fibrous tissue is devoid of elastic fibers. This type of cardiac involvement was similar to that observed in carcinoid heart disease, but it differed in the fact that the deposits were observed on both surfaces of the valve leaflets rather than just on one surface as occurs in the carcinoid condition. The cause of Takayasu's arteritis was uncertain, but the finding of an atypical bacterium in the blood raises the possibility that it is of infection etiology. Monoclonal gammopathy likewise had not been observed previously in a patient with Takayasu's arteritis.

Serial No. -NHI-344 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Proposed Course: This study is accepted for publication in the American Journal of Medicine.

Serial No. -NHI-345 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Pulmonary Parenchymal Cholesterol-ester Granulomas in Patients with Pulmonary Hypertension

Previous Serial Number: None

Principal Investigator: D. Luke Glancy, M. D.

Other Investigators: Paul D. Frazier, D. D. S.
William C. Roberts, M. D.

Cooperating Unit: Laboratory of Histology and Pathology
NIDR

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: Since 1936 it has been appreciated that anatomic changes occur in the lungs of most patients with severe pulmonary hypertension. These changes not only occur in the pulmonary blood vessels, but also in the pulmonary parenchyma. A change, however, that has not been described previously in patients with pulmonary hypertension were granulomas containing acicular crystals in the lungs. During a 12 year period we found these granulomas in the lungs of 12 autopsied patients. Each of the 12 patients had severe pulmonary hypertension. Similar pulmonary granulomas were not observed in any patient studied at necropsy during the same 12 year period who did not have pulmonary hypertension. The crystals which were doubly refractile under polarizing light were dissected out with a fine needle and then they were subjected to diffraction using Z filtered CR radiation. The x-ray diffraction of the pulmonary crystals disclosed that they consisted of cholesteryl palmitate and/or stearate and did not consist of free cholesterol. The cause of the pulmonary ester granulomas, however, was not determined. The fact that they did not occur within blood vessels was evidence that they were not secondary to emboli of atheromatous material. The only common feature among the 12 patients was pulmonary hypertension.

Proposed Course: This study was accepted for publication in the American Journal of Medicine.

Serial No. -NHI-346 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Heart in Malignant Lymphoma (Hodgkin's Disease, Lymphosarcoma, Reticulum Cell Sarcoma and Mycosis Fungoides): A Study of 196 Autopsy Patients.

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: D. Luke Glancy, M.D.
Vincent T. DeVita, M.D.

Cooperating Unit: Medicine Branch, NCI

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: This study was undertaken to determine the incidence of cardiac metastases in patients with malignant lymphoma and to access their clinical significance. Of 196 patients studied at autopsy, 48 (24%) had lymphoma involving the heart. In 27 subjects the cardiac lymphoma was observed on gross examination and in the other 21 patients cardiac lymphoma was found only on the study of histologic sections. Lymphoma in the heart occurred most frequently in mycosis fungoides (33%) and least frequently in Hodgkin's disease (16%). Cardiac lymphoma was found in approximately 25% of patients with lymphosarcoma, reticulum cell sarcoma, and undifferentiated or mixed cell malignant lymphoma. The cardiac metastases, when observed grossly, were usually firm, white, focal nodules located mostly in the pericardium but also often in the walls of the chambers. The incidences of dyspnea, chest pain, effusions into body cavities, precordial murmurs, ventricular gallops, edema, and electrocardiographic disturbances were similar in patients with and without cardiac lymphoma, and these clinical findings most often were the result of mediastinal, pleural or pulmonary lymphoma, anemia, hypoalbunemia, or an underlying cardiac condition. Signs or symptoms of cardiac dysfunction could be attributed to lymphomatous involvement of the heart in only 5 of the 48 patients with cardiac lymphoma. Three of them had mycosis fungoides, and 2, Hodgkin's disease.

Proposed Course: This work has been accepted for publication in the American Journal of Cardiology.

Serial No.-NHI-347 (c)
1. Clinic of Surgery
3½. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Angiosarcoma of the Heart.

Previous Serial Number: None

Principal Investigator: D. Luke Glancy, M.D.

Other Investigators: Jorge B. Morales, Jr., M.D.
William C. Roberts, M.D.

Cooperating Unit: Department of Pathology, Providence Hospital

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: Angiosarcoma is a rare malignant tumor that most often occurs in the skin and subcutaneous tissue, but may also arise from any blood vessel. A patient is described in whom the tumor originated in the heart. It involved extensively the pericardium, and infiltrated the right atrial cavity to partially obstruct blood flow. The patient, who was 35 years old, had been well until two months before death.

Although angiosarcoma of the heart had been described previously, the present report was the first to emphasize that it generally causes obstruction to the right-sided cardiac chambers by hemopericardium with tamponade, by construction of the ventricles from the epicardial tumor, by venal caval or tricuspid valvular obstruction or by a combination of these mechanisms.

Publications:

Glancy, D. L, Morales, J. B., and Roberts, W. C.: Angiosarcoma of the heart. Am. J. Cardiol., 21:413, March 1968.

Serial No. -NHI-348
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Double Orifice Mitral Valve: A Study of the Anomaly in Two Calves and a Summary of the Literature in Humans.

Previous Serial Number: None

Principal Investigator: Jay Rosenberg

Other Investigators: William C. Roberts, M.D.

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: Two mitral orifices of equal size are described in each of two calves, and reports of double orifice mitral valve in 39 human beings are reviewed. Of reported patients with double orifice mitral valve, the malformation occurred as an isolated one in approximately half of them, and in association with other major cardiovascular anomalies in the remainder. The most common associated anomaly is partial persistent common atrioventricular canal. The true developmental basis of double orifice mitral valve is unknown. Although an impressive anatomic malformation, the double orifice appears to cause no disturbance in the function of the mitral valve.

Proposed Course: This study has been accepted for publication in the Archives of Pathology.

Serial No. NHI-349 (c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Processing of Large Blocks of Tissue

Previous Serial Number: None

Principal Investigator: Larry D. Ent

Other Investigators: None

Man Years

Total:	1/6
Professional:	1/12
Other:	1/12

Project Description: A method of processing large blocks of tissue is described. It is not generally recognized among histology technicians and pathologists that it is possible to produce excellent sections from blocks of tissue up to 6 X 5 X 2 cm. in size using standard histopathology equipment. Blocks of tissue of this size are routinely processed in the Section of Pathology, Clinic of Surgery, alongside small sized tissues and the techniques are adjusted to obtain sections of maximum quality as well as quantity. The processing of these blocks of tissue including fixation, dehydration, clearing, infiltration, imbedding and cutting is summarized in this study. The purpose of the large sections is that much information can be obtained from them that cannot be obtained from smaller sections and the techniques which make these larger sections possible are described in this report.

Proposed Course: This report was accepted for publication in the American Journal of Clinical Pathology.

Serial No. -NHI-350
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Tomlinson Fort: Physician and Statesman.

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: None

Man Years
Total: 1/6
Professional: 1/12
Other: 1/12

Project Description: A study is made of a man who was a soldier, statesman, newspaper founder, publisher and editor, bank president, a founder of his state's medical school and of the first state asylum for the insane in the country, distinguished practitioner of medicine for 50 years, and the author of a 736-page medical textbook. This man was Tomlinson Fort who was born in 1787 in a small community in Georgia and died in 1859 in the state's capitol of Milledgeville. The only medical writing that this man produced during his career was the large medical textbook which was printed by his newspaper presses. The book is of considerable interest since it depicts the state of practice in a small Southern community during the first half of the nineteenth century. Studying this book dramatically calls to attention the tremendous progress which has been made during the past 100 years in all fileds of medicine, but particularly the cardiac area. Fort, for example, devoted only 6 pages to diseases of the heart and only 9 to cancer. He stated that heart disease was the least common of all diseases, that angina pectoris is rare, that auscultation was unnecessary. Blood letting, emetics, and cathartics were the therapy for nearly every disease at that time.

Publications:

Roberts, W. C.: Tomlinson Fort of Milledgeville, Georgia: Physician and Statesman. Hist. Med. & Allied Sciences, 23:131, April 1968.

Annual Report of the
Laboratory of Biochemical Genetics
National Heart Institute
1967-1968

The activities of the Section of Molecular Biology center upon the study of molecular mechanisms for the storage and retrieval of information at genetic and neural levels.

Translation and Regulation of the Genetic Message

A Role for tRNA as a Cofactor Coupling Amino Acid and Protein Synthesis:

A new enzymatic pathway for the synthesis of glutamine-tRNA B. megaterium has been found which is dependent upon the formation of a missense AA-tRNA precursor. An amino acid activating enzyme which mismatches amino acid and tRNA, catalyzes glutamic acid acceptance by tRNA responding to glutamine codons. The conversion of missense glutamic acid-$tRNA^{Gln}$ to glutamine-$tRNA^{Gln}$ is catalyzed by a specific amido-transferase and is dependent upon ATP, Mg^{++}, and an amido-nitrogen donor, such as glutamine or asparagine. The reactions are as follows:

a) C^{14}-Glutamic acid + $tRNA^{Gln}$ $\xrightleftharpoons[\text{ATP, } Mg^{++}]{\text{mismatching Glu-tRNA Ligase}}$ C^{14}-Glutamic acid-$tRNA^{Gln}$

b) C^{14}-Glutamic acid-$tRNA^{Gln}$ $\xrightarrow[\text{ATP, } Mg^{++}\text{ GLN or ASN}]{\text{Amido-transferase}}$ C^{14}-Glutamine-$tRNA^{Gln}$

The specificity of the amido-transferase for tRNA is high. The pathway was found in extracts from three of four species of microorganisms examined.

These studies show that $tRNA^{Gln}$ functions in vitro both as a cofactor and as a regulator of the synthesis of glutamine destined for protein.

The Termination of Protein Synthesis:

Oligonucleotides containing known sequences of amino acid and terminator codons were synthesized and used as templates for the synthesis of free and ribosomal bound peptides. The mechanism of codon dependent termination and release of peptides from ribosomes is being studied.

Cellular Differences in mRNA Codon-tRNA Relationships as Potential Regulatory Mechanisms:

Aminoacyl-tRNA preparations from various organisms respond similarly to most, but not all, mRNA codons. We have been particularly interested in the non-universal responses because of the possibility that factors affecting

AA-tRNA serve as mechanisms for regulating the rate of protein synthesis. AA-tRNA was fractionated by column chromatography and codon-anticodon relationships were defined for E. coli and mammalian AA-tRNA corresponding to six amino acids. Mammalian and bacterial AA-tRNA fractions respond to sets of codons according to wobble base pairing. Only seven universal species of AA-tRNA were found with both E. coli and liver AA-tRNA. Seven species of AA-tRNA were obtained from liver that were not detected with E. coli preparations; conversely, five species of AA-tRNA were obtained from E. coli that were not found with liver preparations.

The results indicate that some organisms contain little or no AA-tRNA for certain codons.

Such differences provide potential mechanisms for selectively regulating the rate of protein synthesis and resemble tRNA suppressors that alter codon translation.

Messenger RNA Codon Recognition by Deacylated tRNA and Aminoacyl-tRNA:

Deacylated tRNA has no effect upon the rate of binding of N-formyl-methionyl-tRNA to ribosomes in the presence of initiation factors and GTP. When GTP is omitted, however, tRNA reduces the binding of N-formyl-methionyl-tRNA as expected from the ratio of the deacylated to the acylated tRNA. Current efforts are directed towards elucidating the mode of action of GTP.

Since the rate of translation of messenger RNA may be regulated by changes in tRNA content, we are investigating the AA-tRNA content of embryonic and adult tissues. Several time-dependent differences in AA-tRNA content have been found. Studies in progress are designed to clarify the nature of such differences.

Recognition of the Nonsense Codon, UGA, by a Species of $tRNA^{Cys}$:

Fractionation of cysteine-$tRNA^{E.\ coli}$ by column chromatography yields four partially separated peaks of cysteine-tRNA. Cysteine-tRNA from only one of the four peaks binds to ribosomes in response to UGA, UGC and UGU at relatively low Mg^{++} concentrations. Since UGA may be a terminator codon, the species of cysteine-tRNA which responds to this codon is under investigation to determine whether it functions as a UGA suppressor or as a terminator of protein synthesis.

Regulation of the Rate of Translation of mRNA

Fractionation of Arginine-$tRNA^{E.\ coli}$ by column chromatography revealed three species of Arginine-tRNA which recognize different sets of mRNA codons. One species of Arginine-$tRNA^{E.\ coli}$, approximately 80% of the total Arginine-tRNA, responds to the codons, CGU, CGC and CGA; another species (15%) responds to the codon CGG; the third species (5%) responds to the codons, AGA and AGC.

In E. coli extracts, the rate of protein synthesis directed by randomly-ordered poly AC templates was found to be limited by the concentration of the

AGA-AGG Arginine-tRNA species. The addition of a purified tRNA fraction containing this species of $tRNA^{Arg}$ stimulated protein synthesis 20-fold. Additional information was obtained by determining the relation between the rate of poly U-directed synthesis of polyphenylalanine and molarity of $tRNA^{Phe}$. Maximum incorporation of phenylalanine into protein was observed at a $tRNA^{Phe}$/70S ribosome ratio of 7 to 1. These data show that the rate of translation of different species of messenger RNA may depend upon the frequency of certain codons in messenger RNA and the concentration of the corresponding species of tRNA.

Species of AA-tRNA from Chick Embryos and Adult, Fully-Differentiated Tissues:

Since the rate of translation of messenger RNA may be regulated by changes in tRNA content, we are investigating the AA-tRNA content of embryonic and adult tissues. Several time-dependent differences in AA-tRNA content have been found. Studies in progress are designed to clarify the nature of such differences.

Relation between Allelic State of the Arg^R Locus and Species of $tRNA^{Arg}$ or Arginal-tRNA Synthetase:

The synthesis of the enzymes for arginine synthesis in E. coli is regulated in a non-coordinate manner. To determine whether enzyme synthesis is regulated by an alteration in arginine activating enzymes or by a species of $tRNA^{Arg}$, cell-free protein synthesizing and tRNA preparations have been prepared from R^+ and R^- strains of E. coli. Studies designed to detect possible differences in various components requiring protein synthesis are in progress.

Cytoplasmic, Mitochondrial and Nuclear AA-tRNA:

Transfer RNA preparations from mitochondrial, nuclear and cytoplasmic fractions of guinea pig liver, were converted to AA-tRNA by incubation with AA-tRNA synthetases obtained from whole cells, and were fractionated by reverse phase column chromatography. The nuclear fraction was found to be enriched with respect to AAG-Lys-tRNA, relative to AAA-AAG-Lys-tRNA. In addition, a species of H^3-Gly-tRNA was obtained from the mitochondrial fractions which was not detected with the cytoplasmic fraction. This species of H^3-Gly-tRNA, obtained in high purity, did not bind to ribosomes detectably in response to tri- or polynucleotide codons. No other unusual properties were detected upon further characterization of the Gly-tRNA (H^3-Gly-tRNA was deacylated, the H^3-product was identified, the tRNA was reacylated with H^3-glycine, the chromatographic mobility of the mitochondrial H^3-Gly-tRNA on Sephadex-G_{20} and G_{100} columns was determined). Codon recognition by the mitochondrial Gly-tRNA species has not been detected. The function of mitochondrial Gly-tRNA remains to be determined.

Mechanisms of Neuronal Function:

The genetic approach is being used as a tool to study the function of the nervous system. Attempts are being made to devise methods for selecting behavioral mutants of nematodes and other relatively simple invertebrates. The methods resemble those used for bacterial and Drosophila genetics.

Separation of Various Kinds of Neurons and Glia:

We are trying to devise new methods which may be useful in studies of the nervous system. A systematic survey is in progress to devise methods for dissociating neurons and other cells and separating cell types so that relatively large quantities of each kind of cell will be available for *in vitro* studies.

The activities of the Section on Macromolecules focus on the following problems:

The synthesis and function of the isopentenyl adenosine moiety of tRNA from *Lactobacillus acidophilus* are being studied. Mevalonic acid was shown to serve as a precursor of isopentenyl adenosine. This base was found in tRNA, but not in ribosomal RNA. At least 15% of the isopentenyl adenosine in *L. acidophilus* tRNA is localized in leucine tRNA, and at least 15% in tyrosine tRNA. Smaller amounts of isopentenyl adenosine are found in tryptophan tRNA (5-10%), serine tRNA (2-5%) and cysteine tRNA (2-5%). Essentially all of the mevalonic acid which is incorporated into tRNA is fixed during the early part of logarithmic growth.

The pattern which emerges suggests a definitive correlation of isopentenyl adenosine with the genetic code. Species of tRNA (with the exception of phenylalanine tRNA) that respond to codons that begin with U contain isopentenyl adenosine.

Studies are in progress to determine whether some species of tRNA are derived from other tRNA species by base modification reactions, rather than as a direct gene product.

Serial No.-NHI-351
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A Role for tRNA as a Cofactor Coupling Amino Acid and Protein Synthesis

Previous Serial Number: NHI-179

Principal Investigator: Michael Wilcox

Other Investigator: Marshall Nirenberg

Cooperating Units: None

Man Years:

Total:	3.4
Professional:	1.1
Other:	2.3

Project Description

A new enzymatic pathway for the synthesis of glutamine-tRNA from B. megaterium has been found which is dependent upon the formation of a missense AA-tRNA precursor. An amino acid activating enzyme which mismatches amino acid and tRNA, catalyzes glutamic acid acceptance by tRNA responding to glutamine codons. The conversion of missense glutamic acid-$tRNA^{Gln}$ to Glutamine-$tRNA^{Gln}$ is catalyzed by a specific amido-transferase and is dependent upon ATP, Mg^{++}, and an amido-nitrogen donor, such as glutamine or asparagine. The reactions are as follows:

a) C^{14}-Glutamic acid + $tRNA^{Gln}$ $\xrightleftharpoons[\text{ATP, } Mg^{++}]{\text{mismatching Glu-tRNA Ligase}}$ C^{14}-Glutamic acid-$tRNA^{Gln}$

b) C^{14}-Glutamic acid-$tRNA^{Gln}$ $\xrightarrow[\text{ATP, } Mg^{++}\text{ GLN or ASN}]{\text{Amido-transferase}}$ C^{14}-Glutamine-$tRNA^{Gln}$

The specificity of the amido-transferase for tRNA is high. The pathway was found in extracts from three of four species of micro-organisms examined.

These studies show that $tRNA^{Gln}$ functions in vitro both as a cofactor and as a regulator of glutamine synthesis destined for protein.

903

Honors and Awards: None

Publications:

Brimacombe, R., Kemper, W., Jaouni, T., and Smrt, J.: Oligonucleotidic Compounds. 000+. Protected Derivatives of Guanosine and Adenosine 3'-phosphates. A Synthesis of Diribonucleoside Phosphates Starting from Adenosine and Guanosine Derivatives Bearing a Free NH_2-Group. Czechoslovak Academy of Sciences, in press.

Serial No. NHI-352
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Termination of Protein Synthesis

Previous Serial Number: None

Principal Investigators: C. Caskey, E. Scolnick and R. Tompkins

Other Investigators: M. Nirenberg

Cooperating Units: None

Man Years:

Total:	3.75
Professional:	2.45
Other:	1.3

Project Description

Oligonucleotides containing known sequences of amino acid and terminator codons were synthesized and used as templates for the synthesis of free and ribosomal bound peptides. The mechanism of codon dependent termination and release of peptides from ribosomes is being studied.

Honors and Awards: None

Publications: None

Serial No.-NHI-353
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July, 1967 through June, 1968

Project Title: Cellular Differences in mRNA Codon-tRNA Relationships as Potential Regulatory Mechanisms

Previous Serial Number: None

Principal Investigator: C. Thomas Caskey

Other Investigator: M. Nirenberg

Cooperating Units: None

Man Years:

Total:	0.55
Professional:	0.25
Other:	0.3

Project Description:

Aminoacyl-tRNA preparations from various organisms respond similarly to most, but not all, mRNA codons. We have been particularly interested in the non-universal responses because of the possibility that factors affecting AA-tRNA serve as mechanisms for regulating the rate of protein synthesis. AA-tRNA was fractionated by column chromatography and codon-anticodon relationships were defined for E. coli and mammalian AA-tRNA corresponding to six amino acids. Mammalian and bacterial AA-tRNA fractions respond to sets of codons according to wobble base pairing. Only seven universal species of AA-tRNA were found with both E. coli and liver AA-tRNA. Seven species of AA-tRNA were obtained from liver that were not detected with E. coli preparations; conversely, five species of AA-tRNA were obtained from E. coli that were not found with liver preparations.

The results indicate that some organisms contain little or no AA-tRNA for certain codons.

Such differences provide potential mechanisms for selectively regulating the rate of protein synthesis and resemble tRNA suppressors that alter codon translation.

Honors and Awards: None

Publications:

C. Thomas Caskey, Arthur Beaudet, and Marshall Nirenberg. RNA Codons and Protein Synthesis 15. Dissimilar Responses of Mammalian and Bacterial Transfer RNA Fractions to mRNA Codons. J. Mol. Biol. in press

Serial No. -NHI-354
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Messenger RNA Codon Recognition by Deacylated tRNA and Aminoacyl-tRNA

Previous Serial Number: NHI-183

Principal Investigator: Judith G. Levin

Other Investigator: Marshall Nirenberg

Cooperating Units: None

Man Years:

Total:	1.4
Professional:	1.1
Other:	0.3

Project Description

Deacylated tRNA has no effect upon the rate of binding of N-formyl-methionyl-tRNA to ribosomes in the presence of initiation factors and GTP. When GTP is omitted, however, tRNA reduces the binding of N-formyl-methionyl-tRNA as expected from the ratio of the deacylated to the acylated tRNA. Current efforts are directed towards elucidating the mode of action of GTP.

Honors and Awards: None

Publications:

Judith G. Levin and Marshall Nirenberg. RNA Codons and Protein Synthesis 13. RNA Codon Recognition by Deacylated tRNA and Aminoacyl-tRNA. J. Mol. Biol. in press.

Serial No. -NHI-355
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Recognition of the Nonsense Codon, UGA by a Species of $tRNA^{Cys}$

Previous Serial Number: None

Principal Investigator: Gregory Milman

Other Investigator: M. Nirenberg and C. Caskey

Cooperating Units: None

Man Years:

Total	:	1.05
Professional	:	.75
Other	:	.3

Project Description:

Fractionation of cysteine-$tRNA^{E.\ coli}$ by column chromatography yields four partially separated peaks of cysteine-tRNA. Cysteine-tRNA from only one of the four peaks binds to ribosomes in response to UGA, UGC and UGU at relatively low Mg^{++} concentrations. Since UGA may be a terminator codon, the species of cysteine-tRNA which responds to this codon is under investigation to determine whether it functions as a UGA suppressor or as a terminator of protein synthesis.

Honors and Awards: None

Publications: None

Serial No.-NHI-356
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Regulation of the Rate of Translation of mRNA

Previous Serial Number: NHI-177

Principal Investigator: W. F. Anderson

Other Investigator: M. Nirenberg

Cooperating Units: None

Man Years:

Total:	1.50
Professional:	.60
Other:	.90

Project Description

Fractionation of Arginine-tRNA$^{E.\ coli}$ by column chromatography yielded three species of Arginine-tRNA which recognize different sets of mRNA codons One species of Arginine-tRNA$^{E.\ coli}$, approximately 80% of the total Arginine-tRNA responds to the codons, CGU, CGC and CGA; another species (approximately 15% of the total Arginine-tRNA) responds to the codon CGG; the third species of Arginine-tRNA (approximately 5% of the total) responds to the codons AGA and AGG.

In E. coli extracts, the rate of protein synthesis directed by randomly-ordered poly AG templates was found to be limited by the concentration of the minor species of Arginine-tRNA responding to the codons, AGA and AGG. The rate of poly AG-dependent protein synthesis was stimulated up to 20-fold by the addition of a purified tRNA fraction containing this species of $tRNA^{Arg}$.

Additional information was obtained by determining the relation between the rate of poly U-directed synthesis of polyphenylalanine and molarity of $tRNA^{Phe}$. Below 1.5×10^{-8}M $tRNA^{Phe}$ little incorporation of C^{14} into phenylalanine was detected. At $1.5\text{-}300 \times 10^{-8}$M $tRNA^{Phe}$, the rate of C^{14}-phenylalanine into protein is proportional to $tRNA^{Phe}$ concentration. Maximum incorporation of phenylalanine into protein was observed at a $tRNA^{Phe}$/70S ribosome ratio of 7 to 1.

The data from both in vitro model systems show that the concentration of a species of tRNA can regulate the rate of protein synthesis. The rate of translation of different species of messenger RNA therefore may depend upon the frequency of certain codons in messenger RNA and the concentration of the corresponding species of tRNA.

Honors and Awards: None

Publications:

Serial No. -NHI-357
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Species of AA-tRNA from Chick Embryos and Adult, Full-Differentiated Tissues

Previous Serial Number: None

Principal Investigator: Marshall Nirenberg

Other Investigator: Franklin Portugal

Cooperating Units: None

Man Years:

Total:	1.05
Professional:	0.8
Other:	0.25

Project Description:

Alterations in the chromatographic behavior of tRNA have been observed after viral infection, embryonic differentiation in reticulocytes, and growth of B. subtilis in various media. Since the rate of translation of messenger RNA may be regulated by changes in tRNA content, we are investigating the AA-tRNA content of embryonic and adult tissues. Several time-dependent differences in AA-tRNA content have been found. Studies in progress are designed to clarify the nature of such differences.

Honors and Awards:

Gairdner Foundation Award
National Academy of Sciences
Joseph Priestly Award, Dickinson College
Charles Leopold Award, French Academy of Sciences
N. Y. Academy of Sciences

Publications:

Bernfield, M.R., and Rottman, F.M. Ribonuclease and Oligoribonucleotide Synthesis III. Oligonucleotide Synthesis with 5'-Substituted Uridine 2', 3'-cyclic Phosphates. J. Biol. Chem. 242, 4134, 1967.

Marshall, R., and Nirenberg, M. RNA Codons Recognized by Transfer RNA from Amphibian Embryos and Adults. J. Mol. Biol. in press.

Kano-Sueoka, T., Nirenberg, M. and Sueoka, N.: Effect of Bacteriophage Infection upon the Specificity of Leucine Transfer RNA for RNA Codewords. J. Mol. Biol. in press.

Allende, Jorge E. Protein Synthesis in Wheat Embryos, National Cancer Institute Monograph No. 27: 169, 1967.

Serial No. -NHI-358
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Relation between allelic state of the Arg^R locus and species of $tRNA^{Arg}$ or argininal-tRNA synthetase

Previous Serial Number: None

Principal Investigator: Arthur J. Blume

Other Investigator: M. Nirenberg

Cooperating Units: H. Vogel, Institute of Microbiology, Rutgers University

Man Years:

Total:	0.65
Professional:	0.35
Other:	0.3

Project Description:

The synthesis of the enzymes for arginine synthesis in E. coli is regulated in a non-cordinate manner. To determine whether enzyme synthesis is regulated by an alteration in arginine activating enzymes or by a species of $tRNA^{Arg}$, cell-free protein synthesizing and tRNA preparations have been prepared from R^+ and R^- strains of E. coli. Studies designed to detect possible differences in various components requiring protein synthesis are in progress.

Honors and Awards: None

Publications: None

Serial No. -NHI-359
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Cytoplasmic, Mitochondrial and Nuclear AA-tRNA

Previous Serial Number: None

Principal Investigator: C. Thomas Caskey

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.85
Professional:	0.35
Other:	0.50

Project Description:

Transfer RNA preparations from mitochondrial, nuclear and cytoplasmic fractions of guinea pig liver, were converted to AA-tRNA by incubation with AA-tRNA synthetases obtained from whole cells, and were fractionated by reverse phase column chromatography. The nuclear fraction was found to be enriched with respect to AAG-Lys-tRNA, relative to AAA-AAG-Lys-tRNA. In addition, a species of H^3-Gly-tRNA was obtained from the mitochondrial fraction which was not detected with the cytoplasmic fraction. This species of H^3-Gly-tRNA, obtained in high purity, did not bind to ribosomes detectably in response to tri- or polynucleotide codons. No other unusual properties were detected upon further characterization of the Gly-tRNA (H^3-Gly-tRNA was deacylated, the H^3-product was identified, the tRNA was reacylated with H^3-glycine, the chromatographic mobility of the mitochondrial H^3-Gly-tRNA on Sephadex G_{20} and G_{100} columns was determined). Codon recognition by the mitochondrial Gly-tRNA species has not been detected. The function of mitochondrial Gly-tRNA remains to be determined.

Honors and Awards: None

Publications:

Serial No. -NHI-360
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanisms of Neuronal Function

Previous Serial Number: None

Principal Investigators: M. W. Nirenberg and R. Pertel

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	2.15
Professional:	.85
Other:	1.3

Project Description:

The genetic approach is being used as a tool to study the function of the nervous system. Attempts are being made to devise methods for selecting behavioral mutants of nematodes and other relatively simple invertebrates. The methods resemble those used for bacterial and Drosophila genetics.

Honors and Awards: None

Publications: None

Serial No. -NHI-361
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Separation of various kinds of neurons and glia

Previous Serial No. None

Principal Investigator: Bruce K. Schrier

Other Investigator: M. Nirenberg

Cooperating Units: None

Man Years:

Total:	2.15
Professional:	1.1
Other:	1.05

Project Description:

We are trying to devise new methods which may be useful in studies of the nervous system. A systematic survey is in progress to devise methods for dissociating neurons and other cells and separating cell types so that relatively large quantities of each kind of cell will be available for *in vitro* studies.

Honors and Awards: None

Publications: None

Serial No. -NHI-362
1. Biochemical Genetics
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Isopentenyl adenosine and the structure of tRNA in Lactobacillus Acidophilus

Previous Serial Number: None

Principal Investigator: Alan Peterkofsky

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.4
Professional:	0.6
Other:	0.8

Project Description:

Isopentenyl-adenosine (IPA) is the most recently discovered minor base constituent found in transfer RNA. Thus far, it has only been identified in the tyrosine and serine tRNA's of yeast. In both these cases, there is only 1 mole of IPA per mole of the specific tRNA and the IPA occupies a unique position in the sequence of the RNA. It is, therefore, anticipated that the presence of IPA will confer a special property on these tRNA's.

Our studies in Lactobacillus acidophilus have permitted for the first time the identification of the occurrence of IPA in tRNA in the bacterial kingdom. The choice of this organism for studies on IPA rests on the capacity of the organism to utilize mevalonic acid as a nutritional requirement. We have been able to show with isotope labeling experiments that mevalonic acid serves as the precursor of the isopentenyl group of IPA, and that this base occurs only in transfer RNA and not in ribosomal RNA. The specific labeling during growth with radioactive mevalonic acid of only the rarely occurring IPA provided a unique device for determining the limited number of species of tRNA in L. acidophilus which contain IPA. The specifically labeled tRNA contained a level of C^{14}-IPA consistent with the occurrence of 1 molecule of IPA in 10 to 15% of the tRNA species (i.e., the tRNA for 2 or 3 of the 20 amino acids). The tRNA was enzymatically acylated with a variety of amino acids using a preparation of amino acid activating enzymes derived from lyophilized L. acidophilus. Using a procedure based on the reaction of the free amino group of any amino acid with an amino acid N-carboxyanhydride to form an insoluble polymer, we have been able to achieve partial separations of the tRNA's corresponding to each of a variety of amino acids. A deter-

9.7

mination of the isotope content of these separated fractions allows us to determine if the tRNA corresponding to a particular amino acid contains IPA. This type of analysis has shown that at least 15% of the IPA in the L. acidophilus tRNA is localized in the leucine tRNA and at least 15% in the tyrosine tRNA. Smaller amounts of IPA appear to be in tryptophan RNA (5-10%), serine RNA (2-5%) and cysteine RNA (2-5%). The pattern which emerges from these studies suggests a definitive correlation with the genetic code. All of the tRNA species (except for phenylalanine) whose codons begin with U appear to contain IPA. On the basis of these data we can begin to formulate the sequence requirements for the synthesis of IPA. The relationship of these observations to the structure of the anticodon region and the coding and regulatory properties of IPA will be the focus of our continued experiments in the area.

Honors and Awards: None

Publications:

Peterkofsky, A.: The Incorporation of Mevalonic Acid into the N^6-(^{2}-Isopentenyl)adenosine of Transfer Ribonucleic Acid in Lactobacillus Acidophilus. Biochemistry 7: 742 (1968).

Serial No. -NHI-363
1. Biochemical Genetics
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Interrelationship of Mevalonic Acid and Transfer RNA Metabolism in Lactobacillus Acidophilus

Previous Serial Number: None

Principal Investigator: Marcia Litwack

Other Investigators: Alan Peterkofsky

Cooperating Units: None

Man Years:

Total:	1.15
Professional:	0.95
Other:	0.2

Project Description:

In the past the nutritional requirement of Lactobacillus acidophilus for mevalonic acid has been explained on the basis of its requirement as the precursor of an essential high molecular weight lipid. Our recent studies have shown that of the order of 1% of the mevalonic acid utilized by this organism serves as a precursor of the isopentenyl adenosine (IPA) in the transfer RNA. A series of studies have been aimed at determining the factors which influence the utilization of mevalonic acid for either lipid or nucleic acid synthesis. This could have a bearing on metabolic controls in that organism. In addition, it was anticipated that if a set of conditions were defined whereby the incorporation of mevalonic into transfer RNA was depressed, it might be possible to accumulate a species of tRNA that would serve as a substrate for the enzymatic addition of isopentenyl groups to tRNA.

Measurement of the incorporation of mevalonic acid throughout the growth cycle shows that it does not parallel the bulk of nucleic acid synthesis but rather that essentially all the mevalonic acid which is incorporated into tRNA is fixed during the early part of logarithmic growth. Whether this is due to a shift in the ratio of ribosomal to transfer RNA or a redistribution of the complement of transfer RNA molecules with and without IPA is under further study. A preliminary examination of the ratio of the subspecies of leucine tRNA during the growth cycle has provided some support for this latter possibility.

The effect of variations in concentrations of mevalonic acid presented to the organisms has been investigated. When all other factors are optimal

919

for growth, except that mevalonic acid is limited, the bacteria lyse. This is consistent with the requirement for mevalonic acid for the lipid membrane of the cell, a necessity for maintaining the integrity of the cell. Under one set of conditions which is not yet clearly defined, the cells contain transfer RNA with a lower than normal content of IPA. This tRNA is also characterized by its content of a unique species of leucine tRNA, apparently deficient in IPA, which can be visualized by column chromatography. An enzymatic treatment of such tRNA preparations, in a reaction that requires the addition of isopentenyl pyrophosphate leads to the disappearance of the IPA-deficient tRNA. This, we believe, is the first suggestion of the key reaction in the biosynthesis of IPA, the enzymatic addition of isopentenyl groups to a tRNA acceptor. A further characterization of these observations is in progress.

Honors and Awards: None

Publications: None

Serial No. -NHI-364
1. Biochemical Genetics
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Purification and Properties of Methyl-Deficient Leucine Transfer RNA

Previous Serial Number: None

Principal Investigator: Jonathan Glass

Other Investigators: Alan Peterkofsky

Cooperating Units: None

Man Years:

Total:	0.85
Professional:	0.85
Other:	0

Project Description:

Over the past several years, we have accumulated substantial information on the properties of methyl-deficient leucine transfer RNA. Our evidence supports the idea that methylated bases are essential for fixing the coding properties of these tRNAs. Our present aim is to utilize a variety of column chromatographic techniques to prepare reasonably highly purified sub-species of leucine tRNA, both normal and methyl-deficient. We wish then to study the nature of the interaction of the various sub-species of leucine tRNA with the amino acid activating enzyme. As for the methyl-deficient free species, we expect to detail the chemistry of the deficiency and study in a controlled way the manner in which methylation influences the biological activity.

Utilizing benzoylated DEAE-cellulose, aminoacylated normal tRNA shows three peaks of leucine tRNA, while methyl-deficient tRNA gives four peaks. Depending on the fraction, further resolution of each of the peaks is obtained by chromatography on either MAK or partition columns. Preliminary tests suggest that the purified peak of methyl-deficient leucine tRNA is charged more slowly by the activating enzyme than are the normally methylated fractions. Further study along these lines is in progress.

Honors and Awards: None

Publications:

Capra, J. Donald and Peterkofsky, A.: The Effect of In Vitro Methylation on the Chromatographic and Coding Properties of Methyl-Deficient Leucine Transfer RNA. J. Mol. Biol., 1968, in press.

Serial No. -NHI-365
1. Biochemical Genetics
2. Macromolecules
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Mechanism of Transfer RNA Biosynthesis

Previous Serial Number: None

Principal Investigator: Jonathan Glass

Other Investigators: Alan Peterkofsky

Cooperating Units: None

Man Years:

Total:	.35
Professional	.35
Other	0

Project Description:

This project, recently initiated, is designed to determine if some species of transfer RNA are derived from other species by base modification reactions, rather than as direct gene products. The experimental approach is based on pulse-labeling experiments. Determination of the relative amounts of radioactivity in different purified species of tRNA should allow us to analyze the biosynthetic pathways and learn whether one species of tRNA is the precursor of another.

Honors and Awards: None

Publications: None

ANNUAL REPORT OF THE
LABORATORY OF TECHNICAL DEVELOPMENT
NATIONAL HEART INSTITUTE

ULTRAMICRO METHODS

Microcolorimeters in current use generally require about 2 microliters of sample for 1 cm path absorbiometry. The cuvette is a capillary with fixed or replaceable windows and requires care and time to fill, to eliminate bubbles, and to clean between samples. In addition, great care is necessary to provide reproducible placement of the cuvettes in the optical path of standard or modified spectrophotometers. An effort to divise a simple instrument that would operate with 10 nanoliter samples was undertaken to supply the needs for micropuncture physiological studies.

An instrument based on the idea that fiber optics could be used to conduct the light in and out of a fine glass capillary and at the same time to define the optical absorbance path was constructed. Light from a miniature incandescent lamp filtered by a narrow band interference filter was focused on the large end of a "fiber optics" light guide that was drawn to fit into a capillary of 0.1 mm bore which constitutes the sample cuvette. A similar fiber in the other end of the cuvette defined the light path and conducted the transmitter light to either a solid state detector or a multiplier photo tube.

To fill the cuvette, a transfer pipette was placed at the flared end of the cuvette with the entrance fiber removed but the exit fiber in place. Capillarity of the clean cuvette caused it to fill completely. The entrance fiber was inserted to a preset position and the transmission was measured. At the completion of the measurement, both fibers were disengaged from the cuvette and it was cleaned by suction and water rinsing. In order to insure proper filling, the cuvette had to be dried completely; residual water films rapidly bridged the diameter of the tube and impeded filling. A few seconds of room air suction was adequate to dry the tube.

There was no difference in the linearity of the absorbance-concentration curve between that measured on a Beckman DU and the microcolorimeter over the range of zero to ten micromolar chlorphenol. Samples did not show effects of evaporation until one minute after the cuvette was filled and plugged with the fibers. Precision was better than one per cent including errors of pipetting and fiber placement. The sample volume needed for this unit was about 100 nl. The colorimeter, excluding readout and power supplies, is only 10 x 12 x 6 cm.

We expect to try to reduce the sample volume requirement to 10 nl by using 30 μ fibers and capillaries. In addition, there exist solid state light sources emitting most of their energy in a narrow band in the red part of the spectrum. These, coupled with available sensitive solid state photodetectors would make an extremely compact special purpose unit. A simple spectrophotometer can be made using a multi-layer interference wedge as an adjustable monochrometer. Quartz fibers can be used for ultra-violet

measurements. By mounting the phototube at right angles to the cuvette, the unit can be made into a simple fluorometer or spectrofluorometer. By attaching side arms to the cuvette, a flow system can be constructed which would be useful for routine microcolorimetry.

CAPILLARY TECHNIQUES FOR MICROBIOLOGY AND IMMUNOLOGY

Capillary tubes containing microorganisms or cells in nutrient agar form the basis for several methods for counting and characterizing growth with time and the growth response to additions or immunocellular reactions. Viable bacteria suspended in agar media can be counted by comparing the size of light scattering pulses after successive periods of incubation. Only viable organisms multiply to produce growing colonies that scatter more light with each scan. Similar arrangements of a highly focussed light beam scanning a moving capillary are used to indicate the swelling of cells or the lysis of indicator cells for evidence of cell growth, swelling reaction or lysis.

The capillary tube containing agar and nutrient has been demonstrated to be comparable in accuracy to petri plate methods for counting bacteria in urine and results can be obtained in about 5 hours. Applied to testing for sensitivity to antibiotics, the method has been compared to tube dilution titers and agreement is within one tube; the capillary scanner results are obtained in 5 hours. Commercial prototype instruments from two manufacturers have been used in these tests and both companies intend to market machines for automation of microbiology using our methods.

A method of preloading the capillaries with a known concentration of antibiotic has been worked out and is also useful for preloading the tubes with immunoreactive substances or test reagents. The method involves the deposit of an agar cast from a soft plastic capillary that fits inside the glass capillary. A pair of rollers withdraws the plastic tube, filled with the reagent-contained agar, leaving the cast in the glass tube. When dried, the agar holds the material against the tube wall as an invisible deposit that does not melt when fresh molten agar is drawn into the tube, but holds the additive and permits it to diffuse evenly throughout the diameter of the core.

Pulse height analysis of the repeated scans has been shown in preliminary experiments to resolve the characteristic growth patterns of mixed populations of organisms. An effective pulse height analysis and data handling system is being assembled.

The same method has been tested for counting and determining the size of the zones of lysis of erythrocytes in a modified Jerne technique to quantify the antibody production of spleen cells. The spleen cells to be assayed with complement and erythrocytes are placed in an agar-gel matrix in a capillary which is then passed thru the scanning beam. Clear zones of hemolysis around the active cells are counted and measured in a modified apparatus. These zones or plaques, can be counted and related to the conditions of sensitization, etc. Automatic or semi-automatic counting of the plaques is extremely desirable to obtain good statistics with reduced error.

Preliminary results indicated the feasibility of adding cells, agar, and complement together to eliminate the two step procedure used in the conventional technique. The plaques were usually less than the diameter of the 1.5 mm capillaries used in the microbiological system, so that we tried several sizes to find the most appropriate. Tubes with inside diameter of 0.10 mm and outside diameter of 0.40 were chosen as a compromise between plaque number (small size meant small volume and therefore few plaques) and plaque size. Plaques were easily detected by measurement of light transmission in contrast to the light scattering technique used in the bacteria counter.

A simple tube translating apparatus, light source, and photodetector were assembled as a prototype plaque counter. A synchronous clock motor pulls the tubes past a 50 μ hole which collimates the light from an incandescant lamp. The transmitted light is detected by a phototransistor. When a plaque appears in the light path, the transmitted light increases; the duration of the signal increase is related to the plaque size.

Counts of plaques by machine have agreed within 25% of the counts obtained by hand, using the conventional petri dish preparation. Plaque size distributions are also in reasonable agreement, although the capillaries have one dimensional geometry compared to the three dimensional geometry of the petri dish method. In vitro lymphocyte responses for tissue typing and study of the role of the lymphocyte immune responses has been hamperd because current techniques require utilization of large numbers of cells and do not allow for evaluation of the responses of single cells. The fate of individual lymphocytes in tissue culture, and the possible role of cell to cell contact, can be analyzed directly by suspending the cells in agar before stimulation with antigen or mitogen. By this technique, the lymphocyte response may be measured by direct observation of the individual cells. The inherent possibilities of selective cloning and isolation of individual cells by microdissection techniques after stimulation are also apparent.

Leucocytes, 10^4 to 10^6 per ml, were suspended in 0.5 to 1.0% agar containing nutritive medium and sealed in glass capillary tubes, inside diameter 0.5 mm; many survived and continued to metabolize for 3 to 7 days while single cells were observed and photographed. By a technique similar to that used in an assay of sensitivity to antibiotics, an agar suspension of mitogen or antigen, for stimulating transformation, may be deposited in the capillary lumen before introduction of the agar-cell suspension. In this way, the separated leucocytes are fixed before being exposed to mitogen or antigen, and are restrained from contact with other cells. Hence the isolated single cell may be observed and studied. Serial observation of cells in this system, using phytohemagglutinin, showed morphological changes characteristic of transformation at 48 and 72 hours. Studies of supravital dye uptake, however, indicated that cells in the center of the tube were less viable than those at the ends suggesting that limitation of CO_2 and O_2 exchange was responsible for the cellular injury.

Single cells can be grown in more favorable circumstances in a container of rectangular cross section constructed of slides and cover slips. In this system, the entire surface of the agar may be exposed to the atmosphere in

the incubator and gas exchange is not limited. This configuration is also more favorable optically because the lens effect of the curved capillary tube is avoided and a shorter working distance, suitable for an oil immersion lens, is obtained. Single cells were thus observed serially and photographed under high magnification phase contrast. Viability of cells so cultured was considerably better, as assessed by supravital staining, than in the capillary system. Mixed cell cultures can be studied in such a system without direct contact of the two cell populations by introducting each population in agar separately.

The technique of lymphocyte culture in agar may also be extended for use in a system automating the tedious direct counting of cells necessary in lymphocyte transformation experiments. The capillary apparatus for counting bacteria will be employed for counting transformed cells on the basis of their increase in size. For this procedure, cells may be suspended in the capillary tubes as described. The agar casts may then be expressed and grown in an open dish of culture medium until the time to assess transformation rate. Then, the casts can be replaced in the capillary tubes and counted in the apparatus. This technique will allow for optimal conditions for cell growth but retain the capillary tube configuration which is now the basis for the bacteria counter.

In the examination of test systems for evaluating the capillary scanner to measure cellular responses, the lymphocyte transformation system was studied. Transformation of peripheral blood lymphocytes to blast-like forms under the influence of various stimuli has far reaching implications, both in clinical medicine and in investigation of the delayed hypersensitivity response. Although data on the effects of stimulation are available, there is almost no literature on the mechanism of the phenomenon. Several lines of reasoning led to the hypothesis that the basic mechanism of transformation may be related to cellular injury. To test this hypothesis, the effects of sublethal sonication on short term lymphocyte cultures were determined and compared to the effects of phytohemagglutinin (PHA) in similar cultures. Cells stimulated by sonication or PHA showed similar blastoid alteration, similar subleties of trypan blue staining during the first three days of culture and similar lysosomal patterns in the altered cells. However, cells from sonicated cultures did not have DNA or RNA synthesis rates above those seen in control cultures. In addition, trypan blue staining characteristics indicated a temporarily reversible injury in PHA stimulated cells, but not in sonicated cells. The findings support the concept that the blast-like transformation induced by both PHA and sonication is related to cell damage.

FLUORESCENCE METHODS

In the past few years, this laboratory has shown that our instrumentation can measure corrected emission and excitation spectra, quantum yields, fluorescence lifetimes, and fluorescence polarization. Although these capabilities are of interest in themselves insofar as they show that commerically available equipment is now fairly adequate, we were also interested in applying the instrumentation to biochemically significant problems.

The binding of 1-dimethylaminonaphthalene-5-sulfonamide by bovine erythrocyte carbonic anhydrase (CA) was studied using an electrophoretically pure sample of enzyme. The binding of DNSA by CA studied by quenching, protein ultraviolet fluorescence, or enhancement of DNSA fluorescence showed that only 1 mole of DNSA was bound per mole of protein. The binding constant at neutral pH was $4 \times 10^6\ M^{-1}$. The emitted fluorescence of free DNSA in water was maximal at 580 mμ with a quantum yield of 0.055, but in the complex, DNSA emitted at 468 mμ with a yield of 0.84. From titrations of DNSA itself, it was concluded that part of the blue-shift was due to expulsion of a proton from the $-SO_2NH_2$ group upon binding to the enzyme. The rest of the blue shift was thought to result from binding of the DNSA in a hydrophobic crevice in the protein. Energy transfer calculations were made possible by the finding that excitation of the tryptophans in the protein resulted in fluorescence of DNSA. The surprising result was obtained that 85% of the photons absorbed by the 7 tryptophans are transferred to the single DNSA that is bound. However, the tryptophan fluorescence was quenched only 73% by the DNSA. Therefore, it was concluded that the DNSA was bound closer to the relatively less fluorescent tryptophans. Using Forster's equation, the average distance of DNSA from any given tryptophan was calculated as 16 A.

In contrast, when one DNS group is attached to the enzyme by reacting the enzyme with 1-dimethylaminonaphthalene-5-sulfonyl chloride, the energy transfer is only 10%. Thus, we conclude that in CA, the tryptophans are in the interior of the protein near the sulfonamide binding site, which is also the active site. The tryptophans are quite distant from the surface to which a covalently attached DNS group would be bound. DNSA was shown to inhibit the enzyme activity. Fluorescence depolarization measurements were also performed, and the relaxation times were calculated from the measured fluorescence lifetime and polarization data. These indicated the protein to be very compact and nearly spherical, in agreement with published intrinsic viscosity data.

In the case of serotonin and other related 5-hydroxyindole derivatives, it was found that the anomalous visible fluorescence had some parallels with excited-state phenomena studied by A. Weller and Forster. It seemed likely that the green fluorescence was arising from a protonated form of the excited state of these 5-hydroxyindoles. In other words, absorption of a photon caused the compounds to be stronger bases. At neutral pH, these compounds have a normal ultraviolet fluorescence at about 350 mμ. Titration by acid causes quenching of the normal fluorescence and appearance of the green emission. The titration curves obtained for various derivatives were similar to those obtained by Weller. Also, when the solutions were frozen, the anomalous green fluorescence disappeared, showing that it was dependent on a diffusional process. Phosphorescence spectra were obtained to show that the green emission was not the same as phosphorescence; furthermore, the direct measurements of fluorescence lifetime showed that the green emission had a decay time of the order of 6-7 nanoseconds. Delayed fluorescence in serotonin was observed. All the evidence indicates that the green emission arises from an excited, protonated state of the 5-hydroxyindoles. Such examples of excited-state protonation are still rare. The emission properties were all documented with corrected spectra, quantum yields, and fluorescence decay time measurements.

When a protein combines with a fluorescent substance, the complex has an extrinsic fluorescence which is to be distinguished from the intrinsic protein fluorescence due to aromatic amino acid residues. It is important to study these fluorescent protein complexes because they give information on protein structure; also, fluorescent proteins are used in immunology to localize antigens - nevertheless quantitation of the fluorescence properties has never been adequate. Because we now have the capability of measuring a number of fluorescence of parameters as well as phosphorescence, a number of systems containing proteins plus fluorescent ligand are amenable to description. Among the most important are labeled antibodies or gamma globulins, and complexes of serum albumins.

Dye-labeled protein conjugates are often used for fluorescence depolarization measurements in which it is assumed that the labeling of the protein is random and that dye residues all have the same fluorescence lifetime. These assumptions have never been adequately studied.

A series of conjugates containing the DNS group (1-dimethylaminonaphthalene-5-sulfonyl) attached to serum albumin has been prepared. It was found that the fluorescence spectrum and quantum yield are dependent on the degree of labeling. Thus, the labeling cannot be random. Several fluorescence lifetimes are observed, suggesting that dyes attached at different places have different emission properties. Measurements of fluorescence polarization confirm the presence of different lifetimes. In calculating the degree of labeling of these conjugates, it is usually necessary to assume an extinction coefficient near 340 mμ for the optical absorption of the dye. Since radioactive DNS chloride was available, a study was made to determine the degree of labeling from the radioactivity and thus to check on the reliability of the extinction coefficient usually assumed for DNS.

A method for solubilizing DNS-protein conjugates in a scintillation counting fluid was developed, and the extinction coefficients of DNS on various proteins was determined. It was found that the usually assumed extinction coefficient was about 30% too high for most proteins and that the extinction coefficient varied with different proteins, being especially low in conjugates of ovalbumin.

A number of conjugates were also prepared containing DNS and gamma globulin and fluorescein and albumin or gamma globulin. Measurements of degree of labeling, fluorescence spectra, quantum yields, lifetimes, polarization, and absorption spectra have been made. In fluorescein conjugates, the quantum yields are lower than for free fluorescein, and the dependence on pH is dependent on the degree of labeling. All the data must be evaluated more thoroughly, but it appears that the quantum yields are greatest for the conjugates most lightly labeled; with higher degrees of labeling, dye-dye interaction causes quenching and depolarization of fluorescence. All of these effects are of interest with regard to the meaning of the fluorescence depolarization measurements.

Much effort was expended in an attempt to determine the binding constant of bilirubin with human and bovine serum albumin. The physiological transport vehicle for bilirubin is serum albumin, so the bilirubin-albumin complex is

important. The binding is apparently very tight and cannot be studied by equilibrium dialysis with radioactive bilirubin since the compound is unstable. Using fluorescence quenching, it seemed possible to use low concentrations of protein and bilirubin and obtain dissociation constants. Several trials indicated that the first bilirubin molecule bound to human serum albumin has a dissociation constant of about 10^{-7} M. However, the extremely low concentrations and instability of bilirubin made the precision of the experiments quite poor. Also, it appears that bilirubin may exist in solution as an aggregate, thus invalidating the calculations of dissociation constant which assumes a single species of monomeric bilirubin. Data have been obtained, however, on the absorption spectra of bilirubin - albumin complexes as well as their fluorescence spectra. These data, along with quantum yields, etc., may be of sufficient interest to report.

ARTIFICIAL ORGANS, ASSISTORS, AND MATERIALS PROBLEMS

A membrane artificial lung is currently the method of choice in both long-term and short-term blood perfusions. The membrane lung appears to be indispensable for perfusions of more than an hour in cases of assisted bypass for congestive heart failure, for pulmonary insufficiency in the newborn (hyaline membrane disease), in chronic pulmonary disease, massive bilateral pneumonia or pulmonary infarction, or for use as the respiratory component in the artificial placenta.

The artificial lung is expected to substitute for the major known respiratory functions of the lung, that of O_2 and CO_2 exchange, while not adding to the perfused individual insults from which he cannot recover. The major known injuries associated with artificial lungs that do not use membranes, appear to result from the denaturation of protein and lipid components, mainly from contact with oxygen and surfaces, and secondly from both particulate and gaseous embolization. Medical grade silicone rubber has been used for implantation and as conduits for many years and is relatively inert to tissue fluids and relatively free of cellular reaction. Its high gas permeabilities and availability made it the choice in membrane oxygenators.

During the past year Dow Corning Corporation has delivered several disposable membrane artificial lungs of our design with the capacity of a 300 ml/min. blood flow. These units are being evaluated in collaboration with two pediatric groups, one at Johns Hopkins University and the other at the University of Chicago.

Evaluation at the University of Chicago consists of arterio-venous bypass for periods up to 24 hours in anesthetized puppies. The unit has been shown to have the capacity to supply the total respiratory gas exchange in these puppies. Our Johns Hopkins University associates are carrying out short-term A-V pumping with the membrane lung on anesthetized dogs.

Our own efforts have been confined to A-V perfusion in alert, unanesthetized 108 day old newborn lambs. Immobilization and anesthesia, as well as blood pumping, are known to have significant deleterious effects on both blood and survival rate. By confining our efforts to alert, unanesthetized and unrestrained animals we were able to assess the effects on experimental

animals of long-term A-V perfusion through the membrane lung. Perfusions with long-term survival were carried out for periods up to 91½ hours. Gross pathologic and microscopic examination of organs after elective sacrifice of our long-term survivors showed little or no abnormal findings in any of the organs examined, including lungs.

We have observed, in common with earlier workers, the onset of anemia due to sequestration of red cells in the absence of free hemoglobinemia. Approximately 20% of circulating RBC disappear within 24 hours of perfusion. Donor blood transfusions have been employed with no untoward reactions. Our findings assume significance because of the following findings common in partial perfusions with either the disc oxygenator or the bubble oxygenator: Partial bypass with presently used heart lung devices (disc and bubble oxygenators) in animals has not been successful beyond 48 hours; pulmonary hemorrhage and exudation commences within 2 hours of initiation of perfusion and progresses. Experience accumulated thus far suggests that clinical evaluation of our small membrane lung in patients with severe primary pulmonary disease is justified at this time.

Probe cannulas consititute one important but neglected item in any perfusion device. Cannulas are of crucial importance, since they determine the quantity of blood shunted in an A-V perfusion without a pump. The problem is complex since cannulation of certain vessels (such as the umbilical artery down to the descending aorta) is virtually impossible without a highly flexible and yet imcompressible cannula. Our laboratory has devised a steel-spring-reinforced Lycra cannula which appears to fulfill our requirements. The wall thickness of this novel cannula is kept below that of the thin-walled Teflon or steel cannulae now in use. Our plan includes using these cannulas in A-V oxygenator studies (umbilical artery to umbilical vein).

The small membrane oxygenator (Mini Lung) designed in this laboratory is now commercially available. As of this time it has shown to be suitable for organ perfusion studies. It is too small to be suitable for perfusion in man.

Little further work has been carried out along our early pioneering efforts in mechanically massaging the failing or fibrillating ventricles of the heart. Clinical application of biventricular assistance was carried out elsewhere in over 8 cases, with restoration of blood pressure and re-establishment of normal rhythm and circulation. This included a number of cases where manual cardiac massage had been tried and had proven fruitless.

It has been recently shown that , in animals declared "clinically dead" following exsanguination, if blood is reinfused and the circulation re-established with the "biventricular caridac assistor" the organs (kidney, liver, heart) are maintained in good condition. Organ storage in this fashion has all the advantages, and none of the disadvantages, of organ perfusion in vitro for subsequent transplantation. The method may prove useful for prolonged preservation of human cadaver kidney, heart, or liver for transplantation.

A problem in our study of A-V perfusion through a membrane lung has been our inability to obtain continuous read-out of pO_2 and pCO_2. Intermittent sampling of blood contributes to anemia (especially in premature newborns) and is a cause of potential infection. Recent work by Woldring and associates leads us to believe that mass spectrometry could be used to measure respiratory gas tensions in blood continuously. We have devised a degassing cell to permit continuous sampling of respiratory gases by diffusion through thin walled silicone rubber tubing. Results so far are encouraging.

As organ substitutions require heparinized blood to obviate clotting, there is a need for compatible surfaces and for methods of comparing surfaces. By utilizing reflected light microscopy, thrombogenesis on a variety of surfaces was observed continuously under a controlled flow pattern. The sequence of thrombogenesis was observed and thrombogenicity of various surfaces was studied. A high intensity light source illuminated the blood obliquely through the transparent material that was to be evaluated. A portion of the backscattered light was collected by a microscope objective, and a microscopic image was formed for observation, micrography, or cinematography. Opacity of blood limited the depth of field to 20 to 30 microns so that only the cells adjacent to the material were clearly visible. The developing two dimensional thrombus was observed at the material-blood interface. The material formed the upper protion of a T-tube shaped chamber. A transected canine jugular vein formed the vertical arm of the T. The system was arranged so that only fresh blood previously flowing axially in the vein reached the surface under observation. Blood-flow was controlled with withdrawal pumps and the flows used approximated average flows at the vena caval wall.

Glass, polymethylmethacrylate, siliconized glass, fluorinated ethylene propylene, poly (4-vinyl pyridine) and heparinized poly (4-vinyl pyridine) were evaluated. The conclusions were as follows: 1. The events during the formation of microscopically visible thrombus on a foreign surface are: 1) apparently random deposition of platelets on the surface, 2) isolated aggregation of platelets and, 3) development of a fibrin-RBC mesh between the aggregates.

2. Three patterns of thrombosis were evident: 1) rapidly propagating thrombus developed within 10 minuted in a wake pattern downstream from initiating sites. The predominant thrombus mass was composed of confluent thrombus initiated in wake patterns. 2) On portions of a surface between the flow division and confluent wakes of thrombus, generalized thrombus developed. Thrombus in this area was considered to be initiated by the foreign surface rather than by a contaminant. 3) Downstream thrombus was richer in its content of fibrin and red cells, and had a lower aggregate content than upstream thrombus.

3. Aggregates and interaggregate mesh developed in glass, poly (4-vinyl-pyridine) coated glass, methylmethacrylate, Siliclad coated glass, and FEP film. On poly (4-vinyl-pyridine) heparin coated glass, aggregation occurred, but development of interaggregate mesh and propagating wakes was blunted. GBH coated polymethylmethacrylate windows were free of aggregates at 15 minutes.

4. Methods for evaluating thrombogenicity of materials must avoid placement of the material downstream of any surface initiating thrombosis, in order to

avoid both downstream extension in wake patterns and decrease in proportion of platelet aggregation composing the thrombus mass.

Heparin is widely used to reduce thrombus formation, yet it is not clear what effect heparin has on the platelet aggregation phase as contrasted to the fibrin polymerization phase of thrombosis. This investigation evaluated the effect of heparin on the aggregation and adhesion of platelets to glass and to columns of siliconized glass beads placed in arterio-venous shunts in dogs. The effect of heparin on the aggregation of platelets on glass windows placed in a T-chamber connected to a jugular vein was also studied. The concentration of platelets in blood entering and leaving the columns was determined with a Coulter Counter. The total number of platelets adhering to the column and the percentage removed from the blood in a single pass through the column were calculated at intervals after perfusion began. Flow rates gave calculated average shear rates at the bead surface of zero to twice the shear rate at the vena caval wall. Evaluation of the data showed that increasing heparin concentrations progressively decreased the retention of platelets on the column and that, at a given heparin level, siliconized glass beads caused less retention of platelets than glass beads. When 0.25 or 0.75 mg. heparin/kilogram body weight was used, 95% of the platelets in the blood entering the column were removed by non-siliconized glass in a single pass at 5 minutes. When 27 mg. heparin/kilo were used, the maximum removal was 30% and this was reached at 45 minutes. When siliconized glass beads were used, 2 mg. heparin/kilo resulted in 25% removal at 20 min., and 27 mg. heparin/kilo resulted in less than 10% removal at 45 minutes.

When glass windows were observed at 1/2, 1, or 2 mg. heparin/kilo, both the platelet aggregation and fibrin polymerization phase of thrombosis were markedly reduced, yet even at the 2 mg./kilo dosage (commonly used for cardiopulmonary bypass), platelet aggregates and interaggregate red cell-fibrin mesh developed. However, at 5 mg./kilo, even after 30 minutes, there were no platelet aggregates or fibrin mesh, yet the glass surface was covered with a layer of platelets one cell thick.

While studying adhesion of platelets to various surfaces, we found that sufficient platelets could be removed to produce platelet deficient animals. This simple method of making platelet deficient animals made possible evaluation of platelet production kinetics, and will allow evaluation of the role of platelets in thrombosis.

There are conflicting reports in the literature as to whether or not the venous intima is wettable by aqueous solutions. Since wettability reflects forces of adhesion and indicates chemical constituents at the surface of a material, knowledge of the wettability of intima, may be useful in designing materials which could minimize or prevent thrombus formation when the material was placed in contact with flowing blood.

By examining the contact angle formed by a series of liquids, having different surface tension, when they form air-liquid-intima interfaces, the critical surface tension for the fresh intima can be calculated. The calculated value of 29 to 34 dynes/cm. indicates that the intima should not be wet by water. However, isotonic saline forms an angle of 9° probably

owing to the small molecular dimensions of water that enable it to soak into the intima to form hydrogen bonds. Thus, the intima has a low critical surface tension, yet aqueous media form a very low contact angle on it. Measurement of the contact angle of liquids placed on the intima of dried veins, and od dried veins equilibrated in an atmosphere of 50% relative humidity gave values of critical surface tension of 31-33 dynes/cm.

In the development of perfusion oxygenators the need for accurate blood flow measurements was met with the dual frequency ultrasonic flow meter utilizing high precision phase shift circuits developed for the ultrasonic chromatographic detector described previously. The ease of zero setting and calibration and freedom from drift suggest that the system has greater intrinsic dependability than the magnetic induction system in general use and a cooperative effort with Bell Laboratories and Harvard Apparatus Company is expected to result in a commercial instrument.

In further development of the nuclear magnetic flow meter system it is now apparent that the flowing stream can be magnetically marked by inducing a nuclear magnetization up stream and detecting either the arrival of the marker or by measuring the delivery of a marker in a fashion similar to that in a dye dilution system. The problem of large magnets remains, but is reduced by the lesser need for highly homogeneous fields for this new technique. Whether or not the sensitivity and flow resolution can be made adequate for practical use remains to be determined.

FAST REACTION METHODS

The developments in instruments and methods for the study of biologically interesting fast chemical reactions in solutions include the completion of a commercial version of our stopped-flow apparatus which brings the time range down to 100 microseconds or less. It provides absorption, fluorescence, glass electrode pH, and thermocouple temperature detector systems. In addition a small stopped-flow apparatus commercially available thru the manufacturers of our large instrument has been adapted to a Beckman DU spectrophotometer and the spectrophotofluorometer. The instrument utilizes two air driven tuberculin syringes to deliver solutions into a micro observation tube with a total capacity of 0.2-0.3 ml. The observation tube was a microcuvette modified for the purpose and fixed in a 1 x 1 cm. brass base which fitted directly into the SPF cell compartment. Mixing experiments were done by mixing 4-methylumbelliferone and water in order to determine the dead time. To obtain a low time constant of the photocurrent amplifier, a new transistorized microphotometer was obtained. Data were taken on either a fast pen recorder or a storage oscilloscope. By carefully adjusting the air pressure used to drive the syringes and eliminating all air bubbles in the system, a dead time of about 20 milliseconds was obtained. This should prove useful for a number of reactions of biochemical interest.

The application of the large apparatus to the measurement of the uptake of oxygen by hemoglobin has been of particular interest because of the shape of the curve at the earliest possible observation time and at the highest possible hemoglobin and oxygen concentrations are of importance in the determination of the type of conformational change occuring in the globin. This

in turn is of concern to those exploring various versions of the allosteric theory of enzyme action. Further work in collaboration with investigators at the Cancer Research Institute in Rome, the School of Medicine, University of Alabama, in Birmingham, Alabama, and this laboratory will be carried out during the next year.

A severe test of the system was afforded by the reaction of carbon dioxide and water catalyzed by the red cell enzyme carbonic anhydrase. By increasing the concentration of the enzyme to 400 milligrams per milliter a pseudo first order reaction rate of 1400 sec^{-1} was obtained.

Major problems in cell membrane reactions are analysis of the sequence in which the reactions occur and the energetics of the reactions. By utilizing thermal and optical detectors, the time sequence, kinetics and thermodynamics of the system may be obtained in a single experiment. A simple example of this is in the classical iodine clock reaction in which there are three thermal reactions and one optical reaction. The sequence of reactions when heme and apomyoglobin or aphohemoglibin combine is presently under investigation in cooperation with NIAMD and the sequence of reactions of membrane ATPase in extracted systems is being studied in cooperation with NINDB. Several other such systems such as hormone binding to proteins with NIAMD and sequential enzyme steps with Birmingham are being explored.

During the past year a reliable thermocouple detector system with a one millisecond response time has been brough to commercial development and is presently being used to study various fast reactions. An R and D program is under way with a commercial company to develop reliable fast pH electrodes. Units have been obtained that have response times of less than one millisecond but a number of problems still remain. Considerable testing and evaluation is planned for the next year. A similar situation exists with the fast thermistor detectors; we expect to receive a new unit of our own design by the end of the summer.

The above flow apparatus developments have led to the design and construction of a flow apparatus for the microcalorimeter. This instrument consists of a precision syringe dirve unit which can cause four 10, 5, or 2.5 ml. syringes to deliver 0.100 ± 0.001 ml. of solution each in 0.1 to 5 seconds. A differential mechanical heat of mixing artifact of 10 to 20 microcalories is obtained. A commercial version of the Calvet-Evans-Brown microcalorimeter is under construction and it is expected that the completed stopped-flow microcalorimeter will be ready for testing in the fall. This instrument will be used in pursuing our studies of the red cell ATPase reactions in cooperation with Department of Medicine, Case-Western Reserve University and the DPN/DPNH-TPN/TPNH clock reactions in cooperation with the Department of Biochemistry, School of Medicine, University of Florida.

Serial No. NHI - 366
Lab of Technical Development
Bethesda Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Extracorporeal Oxygenation in Newborn Lambs

Previous Serial Number: None

Principal Investigator: Warren M. Zapol

Other Investigators: Theodor Kolobow, Joseph E. Pierce, R. Levy

Cooperating Units: Dr. A. F. Keeley, Mallory Institute of Pathology, Boston, Massachusetts

Man Years (computed for the 12 month period)

Total: 1.0
Professional: 1.0

Project Description: Clinical testing, gas transfer evaluation, and an analysis of silicone membrane oxygenator effects on red cells and plasma.

Progress During Past Year:

a) Background: A commercially manufactured (Dow Corning Corp.) silicone membrane oxygenator based on Dr. Kolobow's published design has been evaluated in in-vivo testing in 21 newborn lambs. This was carried out while determining the feasibility of prolonged extracorporeal oxygenation in the newborn.

b) Results: Arteriovenous perfusions of from 45-85 cc/min/Kgm. were carried out in alert, normoxic animals for periods up to 96 hours without blood pumps with long term survival post-perfusion. This work demonstrates that such perfusions are compatible with survival and do not cause significant recognizable damage to the perfused animal.

A significant, progressive, sequestering anemia, without the presence of free hemoglobin has been described for the first time. These perfused red cells appear normally on smear, have unchanged CR^{51} half lives, and osmotic fragilities.

Membrane oxygenator efficiency during long term gas transfer, up to 96 hours, has been shown to remain constant in the absence of significant clotting.

Evaluation of the changes occuring to lipoproteins when subjected to a membrane oxygenator devised in this laboratory in contrast to a bubble

935

oxygenator were carried out. A significant increase in phospholipid and cholesterol ester fragments is noted in the analysis of plasma directly exposed to a blood-gas interface. Such fragments are believed to be biologically toxic. These tests were carried out with the cooperative facilities of the Laboratory of Molecular Diseases, NHI, under the guidance of Dr. R. Levy.

c) Direction of Current Research:

Present data supports the conclusion that extracorporeal A-V oxygenation can be pursued to support gas exchange in the neonate with hyaline membrane disease. The production of optimally designed membrane oxygenators will allow us to clinically test this concept. Further observations of the changes in lipoproteins brought about by interface denaturation may allow us to determine the quality of various membrane and disc oxygenators as well as possibly stabilize these important proteins.

Honors and Awards: None

Publications:

1. Kolobow, T., Zapol, W. M., Pierce, J. E., Kelly, A. F., Replogle, R. L., and Haller, A.: Partial extracorporeal gas exchange in alert newborn lambs with a membrane artificial lung perfused via an A-V shunt for periods up to 91 hours. Transactions of the American Society of Artificial Internal Organs, April 21 and 22, 1968.

2. Kolobow, T., Zapol, W. M., and Marcus, J.: Development of a disposable membrane lung for organ perfusion. Appleton Century Croft's Book, Organ Perfusion, 1968. (In press).

Serial No. NHI -367
Lab. of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Development of Apparatus for the Study of Fast Reactions in Biochemical and Cellular System

Previous Serial Number: NHI-207

Principal Investigators: Robert L. Berger, John Kernohan (visiting scientist) Bohdan Balko

Other Investigators: F. J. W. Roughton, L. Rossi-Bernardi, W. Borchardt, W. Friauf, R. Podolsky, R. Schracter, E. Antonini and E. Haddad

Cooperating Units: Department of Colloid Science, Cambridge University.
Institute of Chemical Biology, Univ. of Milan.
Institute of Molecular Biology, Univ. of Rome.
BEI-DRS, LPB-NIAMD, LB-NHI, Science Products Corp.
Instrumentation Lab-Ingold

Man Years (computed for the 12 month period)

Total: 3.0
Professional: 2.5
Other: 0.2

Project Description:

This is a continuation of work described in the PHS-NHI Individual Project Report for fiscal year 1966, Serial No. NHI-207. The large rapid flow system has been utilized in a series of studies on hemoglobin with Antonini. The results of the experiments showed that the reactions with oxygen followed a similar increase of rate with O_2 concentration as does CO. It was not possible to carry out further work in Rome due to leaks occurring in the stop-valve and at the junction of the observation tube and the mixing chamber. These leaks were small enough that they did not show up until critical experimental evaluation was made during operation of the system on a daily basis. Modifications have now been made of the apparatus by Science Products and the new apparatus will be subjected to another series of critical tests. While Science Products will remain the manufacturer of the instrument, the American Instrument Company has taken over marketing and supplying parts. Thus, with the completion of present tests and experiments, the developmental phase of this instrument will be finished. Utilizing an earlier version of the instrument operating at a velocity of 10 m/sec., we have been developing a

stopped flow pH apparatus. A considerable number of technical problems remain but preliminary tests indicate time resolution of 1 millisecond, sensitivity of .001 pH unit, and a 1 ml solution volume per reaction.

The above pH detector can be replaced by a new thermocouple coated with Paralyene C. which has a response of 1 millisecond. This is coupled into an AC coupled amplifier built by Biomedical Engineering which gives a sensitivity of 1×10^{-3} °C at these speeds. Again about 1 ml of solution is needed per reaction. Finally packaging of the system is in progress and experimental testing on various enzyme systems will be carried out during the next year.

Work on the CaEGTA system will be reported in the LPB-NIAMD Annual Report.

Direction of Current Research:

The optical absorption and fluorescence apparatus will be thoroughly checked out on several enzyme systems, namely O_2-CO-Hb, albumin-thyroxan, CaEGTA. The pH and thermal detector systems will be checked out on the CO_2 plus Glyl - glycine and CO_2 plus hemoglobin systems. Model enzyme system experiment using L-amino-acid oxidase, chrymotrypn, and trypsin will be carried on to demonstrate the utility of the method and its limits of usefulness. Work on a micro system using the same instrument principles will be initiated.

Honors and Awards: None

Publications:

Berger, R. L., Balko, B., Borcherdt, W. and Friauf, W.: High Speed Optical Flow Apparatus, Rev. of Scientific Instruments, 39: 486-493, 1968.

Berger, R. L., Balko, B. and Chapman, H. F.: High Resolution Mixer for the Study of the Kinetics of Rapid Reactions in Solution. Rev. of Scientific Instruments, 39: 493-498 (1968).

Berger, R. L., Antonini, E., Brunori, M., Wyman, J. and Rossi-Fanelli, A.: Observations on the Kinetics of the Reactions of Hemoglobin with Oxygen. Journal of Biological Chemistry, 242: 4841-4843 (1968).

Rossi-Bernardi, L. and Berger, R. L.: The Rapid Measurement of pH by the Glass Electrode: the Kinetics of Dehydration of Carbonic Acid at 25° and 37°. Journal of Biological Chemistry, 243, 1297-1302 (1968).

Berger, Robert L.: Combined Calorimetry and Spectrophotometry in Stopped-Flow Measurements. Biochemical Calorimetry, Chap. 11.

Moore, R., Davids, N. and Berger, R. L.: Finite Element Methods in Cell Dynamics, Special Supplement (1968).

Balko, B. and Berger, R. L.: A Direct Finite Element Analysis Method for Particle Mechanics: The Three Body Problem, Ibid.

Balko, B. and Berger, R. L.: Measurement and Computation of Thermojunction Response Times in the Submillisecond Range. Rev. of Scientific Instruments, 39: 498-503 (1968).

Serial No. NHI-368
Lab. of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Development of a Differential Calorimeter for the Study of Biochemical and Cellular Reactions

Previous Serial Number: NHI-208

Principal Investigator: Robert L. Berger

Other Investigators: H. Brown, R. Eckel

Cooperating Units: Department of Biochemistry, Cancer Research Institute, Columbia, Missouri; Department of Medicine, Case Western University School of Medicine.

Man Years (computed for the 12 month period)

Total: 0.6
Professional: 0. 6

Project Description:

This is a continuation of work reported in NHI-208. The development of a stopped flow microcalorimeter has proceeded to the point where the flow system has been constructed and checked out in a Calvet type of calorimeter. Results show that 4 syringes, 10, 5 or 2.5 ml, can be driven together to deliver 0.100 $\pm$ .001 ml of solution in 0.1 to 5 seconds. A differential mechanical heat of mixing artifact of 10 to 20 microcalories was obtained.

Direction of Current Research:

The construction of a commercial version of the Calvet calorimeter modified according to Brown has been started. This will incorporate the flow device. Physical check-out and Red Cell experiments as an operational investigation of the systems capabilities and limitations will be carried out.

Honors and Awards: None

940

Publications:

1. Davids, Norman, Berger, R. L. Simulation method for the design and data correction of calorimeter. Currents in Modern Biology, Special supplement, 1968.

2. Berger, R. L. Calibration and test reactions for microcalorimetry. Biochemical Calorimetry. Chapter 10, Academic Press, H. Brown, ed. In press.

3. Berger, R. L. Computers for calorimetry, Chapter 12, Ibid.

4. Berger, R. L., Chick, Yu Bing F. and Davids, N.: Differential Microcalorimeter for Biochemical Reaction Studies. Rev. of Scientific Instruments, 39: 362-368 (1968).

Serial No. NHI -369
Lab. of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Development of Sensors

Previous Serial Number: NHI-210

Principal Investigators: Robert L. Berger, Bohdan Balko

Other Investigators: Maurice Green, E. Haddad

Cooperating Units: American Red Cross, Instrumentation Labs.,Washington,D.C.

Man Years (computed for the 12 month period)

Total: 0.5
Professional: 0.5

Project Description:

This is a continuation of NHI-210. Work has progressed on a Thermistor type of fast thermal detector but working units have not yet been received. Several other detectors have been looked into but so far only the thermocouple described last year has shown evidence of being a reliable working model. pH sensing units are under development for use in the stopped-flow apparatus, as discussed under that project and several different glasses, diameters, etc., are being investigated. Working units at 1.9 mm diameter and 8-10 mm have been received and tried. In addition, excited state pk shift experiments using a photo-sensitive system are being conducted to determine the maximum rate of response of the electrodes.

Direction of Current Work:

To finish above work in progress.

Honors and Awards: None

Publications:

Balko, B. and Berger, R. L.: Measurement and Computation of Thermojunction Response Times in the Submillisecond Range. Rev. of Scientific Instruments, 39: 498-503 (1968).

942

Serial No. NHI - 370
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Applications of Fluorescence in Biochemistry

Previous Serial Number: NHI - 196

Principal Investigator: Raymond F. Chen

Other Investigators: John C. Kernohan

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total: 0.3
Professional: 0.3

Project Description:

Progress During the Past Year:

a) Background: In the past few years, this laboratory has shown that our instrumentation can measure corrected emission and excitation spectra, quantum yields, fluorescence lifetimes, and fluorescence polarization. Although these capabilities are of interest in themselves insofar as they show that commercially available equipment is now fairly adequate, we were also interested in applying the instrumentation to biochemically significant problems.

Previously, a study was done on the "dansyl" amino acids (derivatives of 1-dimethylaminonaphthalene-5-sulfonyl chloride) in this laboratory in which it was shown that the emission spectrum was markedly sensitive to the dielectric constant of the environment, and in our publication it was suggested that such derivatives would be useful probes to study protein structure. Some work on bovine carbonic anhydrase by Dr. Kernohan showed that sulfonamide inhibitors bound to the enzyme tightly and that the binding constants could be obtained by following the quenching of protein fluorescence. He also found that "dansyl" amide (i.e., DNSA or 1-dimethylaminonaphthalene-5-sulfonamide) formed a peculiar blue fluorescent complex with carbonic anhydrase, so it was proposed to study this complex further.

Another biochemical problem studied was that related to serotonin (5-hydroxytryptamine). In 1955, Udenfriend found that serotonin showed a green fluorescence in acid solutions when irradiated at 280 mμ. The fluorescence was very unusual because the emission peak was separated by nearly 270 mμ from the excitation peak, and because there was no change in optical absorption in going from neutral to acidic pH. The origin of the visible fluorescence has thus remained obscure.

b) Results: The binding of DNSA by bovine erythrocyte carbonic anhydrase (CA) was studied with Dr. Kernohan using an electrophoretically pure sample of enzyme prepared by him. The binding of DNSA by CA studied by quenching of protein ultraviolet fluorescence or enhancement of DNSA fluorescence showed that only 1 mole of DNSA was bound per mole of protein. The binding constant at neutral pH was 4×10^6 M^{-1}. The fluorescence of free DNSA in water emitted maximally at 580 mμ with a quantum yield of 0.055, but in the complex, DNSA emitted at 468 mμ with a yield of 0.84. From titrations of DNSA itself, it was concluded that part of the blue-shift was due to expulsion of a proton from the $-SO_2NH_2$ group upon binding to the enzyme. The rest of the blue shift was thought due to binding of the DNSA in a hydrophobic crevice in the protein. Energy transfer calculations were made possible by the finding that excitation of the tryptophans in the protein resulted in fluorescence of DNSA. The surprising result was obtained that 85% of the photons absorbed by the 7 tryptophans are transferred to the single bound DNSA. However, the tryptophan fluorescence was only 73% quenched by the DNSA. Therefore we concluded that the DNSA was bound closer to the relatively less fluorescent tryptophans. Using Forster's equation, we calculated the average distance of DNSA to any given tryptophan as 16 A.

In contrast, when one DNS group is attached to the enzyme by reacting it with 1-dimethylaminonaphthalene-5-sulfonyl chloride, the energy transfer is only 10%. Thus, we conclude that in CA, the tryptophans are in the interior of the protein near the sulfonamide binding site, which is also the active site. The tryptophans are quite distant from the surface to which a covalently attached DNS group would be bound. DNSA was shown to inhibit the enzyme activity. Fluorescence depolarization measurements were also performed, and the relaxation times were calculated from the measured fluorescence lifetime and polarization data. These indicated the protein to be very compact and nearly spherical, in agreement with published intrinsic viscosity data.

In the case of serotonin and other related 5-hydroxyindole derivatives, it was found that the anomalous visible fluorescence had some parallels with excited-state phenomena studied by A. Weller and Förster. It seemed likely that the green fluorescence was arising from a protonated form of the excited state of these 5-hydroxyindoles. In other words, absorption of a photon caused the compounds to be stronger bases. In neutral pH, these compounds have a normal ultraviolet fluorescence at about 350 mμ. Titration by acid caused quenching of the normal fluorescence and appearance of the green emission. The titration curves obtained for various derivatives were similar to those obtained by Weller. Also, when the solutions were frozen, the anomalous green fluorescence disappeared, showing that it was dependent on a diffusional process. Phosphorescence spectra were obtained to show that the green emission was not the same as phosphorescence; furthermore, the direct measurements of fluorescence lifetime showed that the green emission had decay times of the order of 6-7 nanoseconds. Delayed fluorescence in serotonin was observed. All the evidence thus pointed to the origin of the green emission as being an excited, protonated state

of the 5-hydroxyindoles. Such examples of excited-state protonation are still rare. The emission properties were all documented with corrected spectra, quantum yields, and fluorescence decay time measurements.

c) Future Work: Although the exact structure of the protonated excited-state form of 5-hydroxyindoles is not established, I suspect on the basis of molecular orbital calculations by Dr. Howard DeVoe (National Institute of Mental Health) that the proton goes onto a carbon at position 6. Discussions suggest that the position of protonation can be studied experimentally by nuclear magnetic resonance. I propose to obtain NMR spectra of 5-hydroxyindole in acid and D_2O before and after irradiation; each of the ring protons has a characteristic chemical shift, and the disappearance of a given resonance as a result of light-induced exchange will indicate the position of protonation in the excited state.

Other biochemical problems will be studied as they suggest themselves; other problems are discussed in a separate project description under the title "Fluorescent Complexes of Proteins".

Honors and Awards: None

Publications:

1. Chen, Raymond F., and Kernohan, John C.: Combination of Bovine Carbonic Anhydrase with a Fluorescent Sulfonamide, J. Biol. Chem. 242, 5813, 1967.

2. Chen, Raymond F.: Fluorescence of Protonated Excited State Forms of 5-Hydroxytryptamine (Serotonin) and Related Indoles, Proc. Nat. Acad. Sci. (U.S.), in press.

Serial No. NHI -NHI-371
Lab. of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Fluorescent Complexes of Proteins

Previous Serial Number: None

Principle Investigator: Raymond F. Chen

Other Investigators: None

Cooperating Units: None

Man Years (computed for the 12 month period)

Total: 0.3
Professional: 0.3

Project Description:

Progress During Past Year:

a) Background: When a protein combines with a fluorescent substance, the complex has an extrinsic fluorescence which is to be distinguished from the intrinsic protein fluorescence due to aromatic amino acid residues. It is important to study these fluorescent protein complexes because they give information on protein structure; also, fluorescent proteins are used in immunology to localize antigens- nevertheless quantitation of the fluorescence properties has never been adequate. Because we now have the capability of measuring a number of fluorescence parameters (e. g., quantum yields, corrected spectra, lifetimes, polarization) as well as phosphorescence, a number of systems containing proteins plus fluorescent ligand is amenable to description. Among the most important are labeled antibodies or gamma globulins, and complexes of serum albumins.

Dye-labeled protein conjugates are often used for fluorescence depolarization measurements in which it is assumed that the labeling of the protein is random and that the dye residues all have the same lifetime. These assumptions have never been adequately studied.

b) Results: A series of conjugates containing the DNS group (1-dimethyl aminonaphthalene-5-sulfonyl) attached to serum albumin has been prepared. It is found that the fluorescence spectrum and quantum yield is dependent on the degree of labeling. Thus, in a sense, the labeling cannot be random. Several fluorescence lifetimes are observed, suggesting that dyes attached

946

at different place have different emission properties. Measurements of fluorescence polarization confirm the presence of different lifetimes. In calculating the degree of labeling of these conjugates, it is usually necessary to assume an extinction coefficient for the optical absorption of the dye near 340 mμ. We noted that radioactive DNS chloride was available, so as study was made to determine the degree of labeling from the radioactivity and thus to check on the reliability of the extinction coefficient usually assumed for DNS.

A method for solubilizing DNS-protein conjugates in a scintillation counting fluid was developed, and the extinction coefficients of DNS on various proteins was determined. It was found that the usually assumed extinction coefficient was about 30% too high for most proteins, and that the extinction coefficient varied with different proteins, being especially low in conjugates of ovalbumin.

A number of conjugates were also prepared containing DNS and gamma globulin and fluorescein and albumin or gamma globulin. Measurements of degree of labeling, fluorescence spectra, quantum yields, lifetimes, polarization, and absorption spectra have been made. In fluorescein conjugates, the quantum yields are lower than for free fluorescein, and the dependence on pH is dependent on the degree of labeling. All the data must be evaluated more thoroughly, but it appears that the quantum yields are greatest for the conjugates most lightly labeled, and with higher degrees of labeling, dye-dye interaction causes quenching and depolarization of fluorescence. All of these effects are of interest with regard to the reactive sites on the protein and the implications with regard to the meaning of the fluorescence depolarization measurements.

Much effort was expended in an attempt to determine the binding constant of bilirubin and human and bovine serum albumin. The physiological transport vehicle for bilirubin is serum albumin, so the bilirubin-albumin complex is important. The binding is apparently very tight and cannot be studied by equilibrium dialysis with radioactive bilirubin, since the compound is unstable. Using fluorescence quenching, it seemed possible to use low concentrations of protein and bilirubin and obtain dissociation constants. Several trials indicated that the dissociation constant of the first bilirubin molecule to bind to human serum albumin has a dissociation constant of about 10^{-7} M. However, the extremely low concentrations and instability of bilirubin made the precision of the experiments quite poor. Also, it appears that bilirubin may exist in solution as an aggregate, thus invalidating the calculations of dissociation constant which assumes a single species of monomeric bilirubin. Data have been obtained, however, on the absorption spectra of bilirubin - albumin complexes as well as their fluorescence spectra. These data, along with quantum yields, etc., may be of sufficient interest to report.

947

c) Future Work: It is intended to do a continuing study of a number of dye-protein systems and to report on each separately. Currently, for instance, the DNS-albumin system is being prepared for publication. Subsequently, the data including quantum yields for fluorescein conjugates should be processed.

The large amount of data on the bilirubin complexes must also be evaluated, and the impression is that there is a considerable amount of publishable data present.

Phosphorescence studies on protein-tye conjugates have essentially never been done. One could study the possibility of triplet energy migration in such systems. When singlet dye-dye energy transfer takes place, what is the effect on triplet luminescence? If the dye-dye interaction is of a certain type, we might expect triplet excimer luminescence or dimer phosphorescence, or triplet annihilation and delayed fluorescence; such possibilities depend on the nature and position of the dyes and should be interesting to study.

Honors and Awards: None

Publications:

1. Chen, Raymond F.: Dansyl Labeled Proteins: Determination of the Extinction Coefficient and Number of Bound Residues with Radioactive Dansyl Chloride, Analytical Biochemistry, in press.

Serial No. NHI-372
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title Methodology in Fluorescence Measurements

Previous Serial Number: NHI-197

Principal Investigator: Raymond F. Chen

Other Investigators: None

Man Years (computed for the 12 month period)

Total: 0.4
Professional: 0.4
Others: None

Project Description:

Progress During Past Year:

a) Background: Some investigators in the field of fluorescence spectrometry have in the past stated that it is necessary to build one's own instrumentation in order to obtain reliable results. We have felt that commercially available apparatus has improved gradually so that the usefulness of such instruments would be increased if methods and techniques for their effective use were devised and reported. In the past we have described methods of obtaining fluorescence polarization, corrected spectra, quantum yields, and fluorescence lifetimes with commercially available equipment. Further tests of instrumentation were continued, especially with regard to a phosphorescence attachment and a stopped-flow attachment for the Aminco-Bowman spectrophotofluorometer. The properties of compounds used in fluorescence as quantum yield standards and pH indicators were also studied, since they are important in terms of methodology.

b) Results: A stopped-flow attachment made by Science Products, Dover, New Jersey was obtained by Dr. R. L. Berger of this laboratory and its usefulness in the fluorometer was tested. The instrument utilizes two air driven tuberculin syringes to deliver solutions into a micro observation tube with total capacity of 0.2-0.3 ml. The observation tube was actually a microcuvette modified for the purpose and fixed in a 1 x 1 cm brass base which fit nicely into the Aminco cell compartment. Mixing experiments were done by mixing 4-methylumbelliferone and water in order to determine the dead time. To obtain a low time constant of the photocurrent amplifier, a new transistorized microphotometer was obtained from Aminco, and the data were obtained with either a recorder or a storage oscilloscope. By carefully

adjusting the air pressure and eliminating all bubbles in the system, a dead time of about 20 msec was obtained, and this is probably adequate for the vast majority of fast biochemical reactions of interest.

An Aminco phosphoroscope attachment was obtained. Phosphorescence spectra were obtained, and there seemed to be no serious problems involved. Some of the spectra were used in a study of serotonin fluorescence to show that the phosphorescence spectrum was different from an anomalous visible fluorescence band. Preliminary testing of the phosphorescence attachment included obtaining spectra of proteins and small organic molecules with a view towards obtaining experience in these arrangements.

About 2 years ago, we noted that the quantum yields of tryptophan and tyrosine were much lower than the values reported by Teale and Weber in 1959. The values we obtained were 0.13 and 0.14, respectively, instead of the reported 0.20 and 0.21. Our initial attempt to report this was met with a rejection from Anal. Chem. in 1965 on the basis that the data were of marginal importance, and it was not clear who was right. However, tryptophan is frequently used as a quantum yield standard, and our methods of determining quantum yield have now been reported in detail. Therefore, the data were submitted and published in Analytical Letters, a new journal.

In looking for a fluorescent pH indicator which could be used to follow rapid pH changes in a stopped-flow fluorometer we studied 4-methylumbelliferon Titration in water showed that the absorption spectrum changed with a mid-point at pH 7.6. Both the neutral and the anionic forms have a blue fluorescence with quantum yields of about 0.7 in each case. One can follow an increase or a decrease in fluorescence on titration with acid or base simply by choosing the right wavelength of excitation. The corrected spectra and lifetimes of 4-methylumbelliferone were obtained.

c) Future Work: The description of the stopped-flow apparatus is in progress. It is also intended to use the apparatus for certain interesting problems in protein denaturation and in studying the rate of combination of various ligands to different proteins.

We intend to continue to obtain experience with the phosphorescence measurements since this type of luminescence is also important in understanding the ways in which an excited state can dissipate its energy. Application to dye-protein systems is attractive, since there is evidence that triplet energy transfer may be a way to study intermolecular distances with molecules which phosphoresce but do not fluoresce.

Honors and Awards: None.

Publications: Chen, Raymond F.: Fluorescence Quantum Yields of Tryptophan and Tyrosine, Anal. Letters 1, 35 (1967).

Chen, Raymond F.: Fluorescent pH Indicator: Spectral Changes of 4-Methylumbelliferone, Anal. Letters.

Serial No. NHI-373
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effect of Heparin on Platelet Aggregation and Adhesion

Previous Serial Number: None

Principal Investigator: Robert C. Dutton

Other Investigators: None

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total: 0.1
Professional: 0.1

Project Description:

Heparin is widely used to prevent fibrin polymerization and to reduce thrombus formation, yet it is not clear what effect heparin has on the platelet aggregation phase of thrombosis as contrasted to the fibrin polymerization phase of thrombosis. This investigation evaluated heparin effect on platelet adhesion and aggregation to glass and siliconized glass bead filled columns placed in dogs' arterior-venous shunts, and the effect of heparin on platelet aggregation on glass windows placed in a T-chamber connected to a jugular vein(report Thrombus Formation on Foreign Surfaces).

Platelet concentration in blood entering and leaving the columns was obtained with a Coulter Counter. The total number of platelets adhering to the column and the % removed from the blood in a single pass through the column were calculated at 5, 10, 15, 20, 30, 45, 60 min after perfusion began. The percent platelets removed in a single pass was calculated as:

$$\frac{\text{(platelet concentration in blood entering column)} - \text{(Platelets concentration in blood leaving the column)}}{\text{(Platelet concentration in blood entering the column)}}$$

Flow rates gave calculated average shear rates at the bead surface of zero to 2X the shear rate at the vena caval wall.

Progress During the Past Year:

Evaluation of the data showed that increasing heparin concentrations progressively decreased platelet retention on the column, and that a given heparin level siliconized glass beads caused less platelet retention than glass beads. When 0, ¼, or 3/4 mg. heparin/kilogram body weight were used,

'151

95% of the platelets in the blood entering the column were removed in a single pass at 5 minutes. When 2 to 3 mg. heparin/kilog were used 95% of the platelets were removed at 10 minutes; when 20 mg heparin/kilog were used 95% of the platelets were removed at 20 minutes. When 27 mg. heparin/kilog were used, the maximum removal was 30% and this was reached at 45 minutes. When siliconized glass beads were used, 2 mg. heparin/kilog resulted in 50% removal at 10 minutes, 20 mg. heparin/kilo resulted in 25% removal at 20 min, and 27 mg. heparin/kilo resulted in less than 10% removal at 45 minutes.

When glass windows were observed at ½, 1, or 2 mg. heparin/kilog, both the platelet aggregation and fibrin polymerization phase of thrombosis were markedly reduced, yet even at the 2 mg/kilog dosage (commonly used for cardiopulmonary bypass), platelet aggregates and interaggregate red cell-fibrin mesh developed. However, at 5 mg/kilog, even after 30 minutes, there were no platelet aggregates or fibrin mesh, yet the glass surface was covered with a layer of platelets one cell thick.

Direction of Current Research: None

Honors and Awards: None

Publications: None

Serial No. NHI - 374
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A New Method of Making Platelet Deficient Animals

Previous Serial Number: NHI - 188

Principal Investigators: Robert C. Dutton

Other Investigators: Robert L. Decrick
Brian Bull

Cooperating Units: BEIB, CC:CP, Department of Hematology

Man Years: (computed for the 12 month period)

Total: 0.1
Professional: 0.1
Other: None

Project Description:

While studying platelet adhesion to carcoal granule or glass bead filled columns placed in a heparinized dog's or cat's arteriovenous shunt, we found that sufficient platelets could be removed to produce platelet deficient animals. This simple method of making platelet deficient animals allowed evaluation of platelet production kinetics, and will allow evaluation of the role of platelets in thrombosis. In dogs sequential placement of 3 columns, each containing 250 cc. of 0.5 mm beads, reduced the mean platelet count from 230,000 platelets mm^3 of blood, to 25,000 platelets/mm^3 of blood. Using glass bead columns, we prepared 4 platelet deficient dogs and maintained them platelet deficient for 3 to 5 days, and studied platelet adhesion of freshly released platelets and the rate of platelet production.

Progress:

1) On the first day, platelets removed by the 3rd column, when the platelets count was 40,000 platelets/mm^3, were as adhesive as platelets removed on the first column when the platelet count was 230,000 platelets/mm^3, indicating the entire population of platelets may adher to the beads.

2) A single column placed in the animal's arterio-venous shunt on the 2nd, 3rd, 4th, or 5th days removed platelets that had been replaced into the blood over the previous 24 hours and brought the platelet count from 60,000 platelet/mm^3 back down to 15,000 platelets/mm^3.

3) These freshly released platelets on the 2nd, 3rd, 4th, or 5th days did not differ in adhesiveness from those present on the first day when the count started at 230,000 platelets/mm^3.

4) The daily rate of production was increased on the 5th day as compared to the 2nd or 3rd day.

Direction of Current Research:

Evaluate thrombogenesis on foreign surfaces in a platelet deficient animal.

Honors and Awards: None

Publications: None

Serial No. NHI -375
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Thrombus Formation on Foreign Surfaces

Previous Serial No.: NHI - 189

Principal Investigator: Robert C. Dutton

Other Investigators: Robert Dedrick

Cooperating Units: BEIB

Man Years

Total: 0.45
Professional: 0.45

Project Description:

By utilizing reflected light microscopy, thrombogenesis was continuously observed on a variety of surfaces, under a controlled flow pattern. The sequence of thrombogenesis was observed and thrombogenicity of surfaces was compared.

A high intensity light source illuminated the blood obliquely through the transparent material that was to be evaluated. A portion of the backscattered light was collected by a microscope objective, and a microscopic image was formed for observation, micrography, or cinematography. Opacity of blood limited the depth of field to 20 or 30 microns so that only the cells adjacent to the material were clearly visible. The two dimensional developing thrombus was observed at the material-blood interface.

The material formed the upper portion of a T-tube shaped chamber. A transected canine jugular vein formed the vertical arm of the T. The system was arranged so that only fresh blood previously flowing axially in the vein reached the surface under observation. Blood-flow was controlled with withdrawal pumps, and the flows used in these experiments approximated average flows at the vena caval wall.

Glass, polymethylmethacrylate, siliconized glass, fluorinated ethylene propylene, poly (4-vinyl pyridine) and heparinized poly (4-vinyl pyridine) were evaluated. The conclusions of this experiment were as follows:

1. The events of microscopically visible thrombus formation at venous shear rates on a foreign surface are: 1) apparent random deposition of platelets on the surface, 2) isolated aggregation of platelets and, 3) development of a fibrin- RBC mesh between the aggregates.

455

2. Three patterns of thrombosis were evident: 1) downstream from initiating sites, often identified as contaminant particles, rapidly propagating thrombus in a wake pattern developed within 10 minutes. The predominant thrombus mass on the surfaces was composed of confluent thrombus initiated in wake patterns. 2) On portions of a surface between the flow division and confluent wakes of thrombus, generalized thrombus developed. Thrombus in this area was considered to be initiated by the foreign surface rather than a contaminant. 3) Downstream thrombus composition increased in fibrin-red cell content and decreased in aggregate content as compared to upstream thrombus.

3. Aggregates and interaggregate mesh developed on glass, poly (4-vinyl-pyridine) coated glass, methylmethacrylate, Siliclad coated glass, and FEP film. On poly (4-vinyl -pyridine) heparin coated glass, aggregation occurred but development of interaggregate mesh and propagating wakes was blunted. GBH coated polymethylmethacrylate windows were free of aggregates at 15 minutes.

4. Methods for evaluating thrombogenicity of materials must avoid placement of the material downstream of any surface initiating thrombosis, in order to avoid both downstream extension in wake patterns and decrease in proportion of platelet aggregation comprising the thrombus mass.

Direction of Current Research:

Evaluation of the effect of monolayers of varying critical surface tension on thrombogenesis.

Honors and Awards: None

Publications: Dutton, R. C., Baier, F. E., Dedrick, R. L. and Bowman, R. L.: Initial Thrombus Formation on Foreign Surfaces. Trans. Amer. Soc. Artif. Int. Organs, In press.

Serial No. NHI -376
Lab. of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Wettability of the Venous Intima

Previous Serial No.: None

Principal Investigator: Robert C. Dutton

Other Investigators: Robert E. Baier (Guest in LTD)

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total: 5 days
Professional: 5 days

Project Description:

There are conflicting reports in the literature as to whether or not the venous intima is wettable by aqueous solutions. Since wettability reflects forces of adhesion and indicates chemical constituents at the surface of a material, knowledge of the wettability of intima, may be useful in designing materials which could minimize or prevent thrombus formation when the material was placed in contact with flowing blood.

By examining the contact angles formed by a series of liquids, having different surface tensions, when they form air-liquid-intima interfaces, we calculated the critical surface tension for the fresh intima. The calculated value of 29 to 34 dynes/cm. indicates the intima should not be wet by water. However, isotonic saline forms an angle of 9° probably due to small molecular dimensions of water enabling it to soak into the intima and hydrogen bond. Thus, the intima has a low critical surface tension, yet aqueous media forms a very low contact angle on it. Measurement of contact angle of liquids placed on the intima of dried veins, and of dried veins equilibrated in an atmosphere of 50% relative humidity resulted in critical surface tension of 31-33 dynes/cm.

Direction of Current Research: None

Honors and Awards: None

Publication: None

Serial No. NHI - 377
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Title: Design and Evaluation of Artificial Internal Organs for Augmentation or Permanent Replacement.
Previous Serial Number: None

Principal Investigator: Theodor Kolobow

Other Investigators: Warren M. Zapol, Robert L. Bowman, Jeffrey Marcus, Gerald G. Vurek

Cooperating Units: Johns Hopkins University, University of Chicago

Man Years: (computed for the 12 month period)

Total: 2.5
Professional: 2.5
Other: None

Project Description:

A. A membrane artificial lung is currently the method of choice in both long-term and short-term blood perfusions. The membrane lung becomes indispensable for long-term perfusions of more than an hour in cases of assisted bypass for congestive heart failure, for pulmonary insufficiency in the newborn (hyaline membrane disease) or the chronically diseased lung, the acutely diseased lung as exemplified by massive bilateral pneumonia or pulmonary infarction, or in the use as the respiratory component in the artificial placenta.

The artificial lung first and foremost is expected to substitute for the major known respiratory functions of the lung, that of O_2 and CO_2 exchange, while not adding insults to the perfused organisms from which it cannot recover. The major known deleterious injuries associated with artificial lungs not using membranes appear to lie in the denaturation of protein and lipid components, mainly from contact with oxygen and surfaces, and secondly from both particulate and gaseous embolization. Medical grade silicone rubber has been used for implantation and as conduits for many years and was shown to be relatively inert to tissue fluids and relatively free of cellular reaction. Its high gas permeabilities and availability in this membrane made it the choice in membrane oxygenators.

During the past year Dow Corning Corporation has begun delivering us duplicate 300 cc blood oxygenating capacity disposable membrane artificial lungs of our own design. These units are being evaluated in collaboration with two pediatric groups, one at Johns Hopkins University, and the other

95

at the University of Chicago.

Evaluation at the University of Chicago consists of A-V bypass in anesthetized puppies for periods up to 24 hours. The unit has proven being able to totally supply respiratory gas exchange in these puppies at selected times.

Our Johns Hopkins University associates are carrying out short-term V-A pumping with the membrane lung on anesthetized dogs.

Our own efforts have been confined to A-V perfusion in alert, unanesthetized 1-8 days old newborn lambs. Both immobilization and anesthesia as well as blood pumping are known to be associated with significant deleterious effects on both blood and survival rate of an animal. By confining our efforts to alert, unanesthetized and unrestrained animals we were able to assess the effects of long-term A-V perfusion through the membrane lung on experimental animals. Perfusions with long-term survival were carried out for periods up to 91½ hours. Gross pathologic and microscopic examination of organs after elective sacrifice of our long term survivors showed little or no abnormal findings in all organs examined, including lungs.

We have observed, in common with earlier workers, the onset of a sequestering anemia in the absence of free hemoglobinemia. Approximately 20% of circulatory RBC disappear within 24 hours of perfusion. Donor blood transfusions have been employed with no untoward reactions.

Our findings assume significance becasue of the following findings common in partial perfusions with either the disc oxygenator of the bubble oxygenator: 1. Partial bypass with presently used heart lung devices (disc and bubble oxygenators) in animals has not been successful beyond 48 hours. 2. Pulmonary hemorrhage and exudation in animal perfusions commences within 2 hours of initiation of perfusion, and relentlessly progresses onward.

Knowhow and experience accumulated thus far makes us believe that clinical evaluation of our small membrane lung in patients with desperate primary pulmonary disease is justified at this time. We are ready to apply it clinically.

B. One important but neglected item in any perfusion device involves that of proper cannulas. Cannulas are of crucial importance, and are the limiting factor that determines quantity of blood shunted in an A-V perfusion without a pump. The problem becomes somewhat more complex when one realizes that cannulation of certain vessels (such as the umbilical artery down to descending aorta) is virtually impossible without a highly flexible and yet incompressible cannula.

Our laboratory has devised a steel spring reinforced Lycra cannula which appears to fulfill our requirements. Wall thickness of this novel cannula is kept below that of thin-walled Teflon or steel cannulae now in use.

959

Our plan includes using these cannulas in A-V oxygenator studies (umbilical artery to umbilical vein).

C. The small membrane oxygenator (Mini Lung) designed in this laboratory is now commerically available. As of this time it has shown to be suitable for organ perfusion studies. It is not suitable for perfusion in man.

D. Work on blood pumps has been initiated in this laboratory. Many bypass pumps implanted intrathoracically were shown to be relatively inocuous (Bernhard, Pierce, DeBakey). This cannot be said of extracorporeal bypass pumping. Anemia with hemoglobinemia is an early persistent and limiting finding.

A thin walled Lycra roller pump tubing was made to permit a totally non-occlusive setting in the roller pump. Present roller pump tubing is heavy walled and does not permit controlled non-occlusive setting of the roller pump.

In collaboration with Litton Industries, molded power actuated tricuspid valves of our own design are to be made available to us for testing. We feel that a ventricular pump using our power actuated valves may be superior to roller pumping as it mimics the function of the natural heart.

E. Little further work has been carried out along our early pioneering efforts in mechanically massaging the failing or fibrillating ventricles of the heart. Our available time was devoted to work on A-V oxygenation in lambs. Work on the "biventricular cardiac assistor" was stalled totally because of the lack in manpower.

In spite of this, other researchers in this field have accomplished significant results:

a). Clinical application of biventricular assistance was carried out in over 8 cases, with restoration of BP and re-establishment of normal rhythm and circulation. This included a number of cases where normal cardiac massage tried beforehand was fruitless. These data suggest that mechanical cardiac massage has been under-rated in the past. Further intensive support by organizations having a mandate in this field (Myocardial infarction program) would be in order.

b). It has been recently shown that animals first exanguinated, declared clinically dead, then blood reinfused and circulation re-established with the "biventricular cardiac assistor" do preserve their organs (kidney, liver, heart) in excellent shape. Organ storage in this fashion has all the advantages, and none of the disadvantages, over organ perfusion in vitro for subsequen transplantation. The utility of this method for prolonged human donor cadaver kidney, heart, or liver preservation suggests itself immediately.

F. Of important concern to our ongoing study in A-V perfusion through a membrane lung has been our inability to continuously read out respective

pO_2 and pCO_2 data. Intermittent sampling of blood contributes to anemia (especially in premature newborns) and is a source of potential infection. Recent work by Woldring and associates leads us to believe that mass spectrometry could be used to measure continuously respiratory gas tensions in blood. We have devised a degassing cell to permit continuous sampling of respiratory gases by diffusion through a thin walled silicone rubber tubing. Results so far are encouraging.

C. Up to now but little progress has been made to have small artificial kidneys fabricated to our specifications. Efforts are continuing to interest outside manufacturers to further develop these units and make them available for clinical testing.

Direction of Current Research:

1. We will seek clinical experience in the use of the membrane lung in treatment of respiratory gas exchange deficits in man. Our major efforts will be confined to this field. Cannula development work and work on continuous respiratory blood gas analysis are an integral part of this project.
2. Subject to manpower and time limitations work will continue on A) blood pump, B) power actuated heart valve, C) biventricular cardiac assistor, D) disposable artificial kidney.

Honors and Awards: None

Publications:

1. Kolobow, T., Zapol, W., Pierce, J. E., Keeley, A. F., Replogle, R. L., and Haller, A. Partial extracorporeal gas exchange in alert newborn lambs with a mambrane artificial lung perfused via an A-V shunt for periods up to 91 hours. Proceedings of the American Society for Artificial Internal Organs. June, 1968.

2. Kolobow, T., Zapol, W. M., and Marcus, J.: Development of a disposable membrane lung for organ perfusion: A. mini-lung B. the spiral coil lung. Appleton Century Crofts. Fall, 1968. In press.

Serial No. NHI - 378
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Nuclear Magnetic Resonance Flowmeter

Preveious Serial Number: NHI - 171

Principal Investigators: Vsevolod Kudravcev
Robert L. Bowman

Other Investigators: None

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total: 1.0
Professional: 1.0
Other: None

Project Description:

Objective: We wish to apply some of the principles and methods of Nuclear Magnetic Resonance to the measurement of blood flow. Specifically the NMR absorption method may, in theory, be useful for measurement of volume flow rate. A new method known as Magnetic Labeling promises to be useful for measurement of flow velocity. This latter method may be of interest to physiologists because it is theoretically possible to detect blood flow and vessel volume changes with the detecting probe located outside of the body. This is possible because the NMR signal from the blood stream is magnetically distinguishable from other protons distributed around the vessels.

Methods Employed: The NMR absorption method described in the previous Individual Progress Report NHI - 171 has been redesigned, rebuilt, and tested with liquids having relaxation times in the same range as that of blood.

A new technique based on the method of magnetic labeling has been developed to the operating stage.

Major Findings: The N.M.R. absorption method yields a reasonably linear (i.e. $\pm$ 3%) and reproducible indication of blood volume flow rate over the restricted range of approximately 5 c.c./sec., e.g. 0-5 c.c., 5-10 c.c., etc. A particular flow cell geometry and r.f. level is required for each range. The new electronic circuit automatically adjusts the r.f. level to achieve best sensitivity and linearity for a given flow probe. Over the range from zero to 15 c.c./sec. the physical size of the probes ranges from 1 m.m. inside diameter x 3 m.m. long to 7 m.m. I.D. x 7 m.m. long. The blood was

962

simulated (in respect to NMR relaxation time) by the addition of varying amounts of Manganese sulfate, a paramagnetic salt. The variation in flow response with changes is salt concentration was studied. It was found that best linearity occurs when the relaxation time is shorter than that of normal blood, although satisfactory linearity can be obtained with blood. The signal to noise ratio averages 25 and the accuracy of the volume flow measured by absorption NMR averages 2½% as compared with stop-watch and rotameter standards. In addition, the stability of the indication is greatly improved over previous models due to refinements in the electronic design and execution.

The Magnetic Labeling method utilizes the fact that time is required for the proton polarization alignment to become random. A strong magnet located upstream is used to strongly polarize the protons in the flowing liquid. A much weaker magnet and NMR probe are located downstream. The presence of polarized protons in the downstream detector is indicated by an increase in its output amplitude. Hence, if the upstream magnet is pulsed on briefly, an increased amplitude will occur at the detector, delayed by the time required by the liquid to flow between the magnets. If this time is measured and the tube geometry is known, the volume flow rate can be calculated.

An alternative system consists of the arrangement above with the addition of an r.f. coil positioned around the tube intermediate between the two magnet assemblies. Pulsing of the r.f. coil results in variations in amplitude and even reversals in polarity (or phase) of the detector output. As before, the geometry and delay time amy be used to obtain the volume flow rate. Successful experiments have been performed in the flow range of 6-10 c.c./sec.

Significance to Bio-Medical Research and the Program of the Institute: The volume flow rate of blood cannot be obtained by the use of flowmeters currently available. The NMR Absorption flowmeter is theoretically capable of indicating volume flow rates, and has been shown to be promising experimentally. The Magnetic Labeling technique is inherently able to measure average flow velocity between two points along a tube. From the geometry and the assumption of a flat flow profile it is possible to obtain the volume flow rate.

Proposed Course: Development will proceed on both the N.M.R. Absorption and the Magnetic Memory systems in an effort to improve the performance of these systems for measuring volume flow rates in conduits.

Honros and Awards: None

Publications: None

963

Serial No. NHI - 379
Lab. of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Dual-Frequency Ultrasonic Flowmeter

Previous Serial Number: NHI - 200

Principal Investigator: Frank W. Noble

Other Investigators: None

Cooperating Units: Bell Labs and Harvard Apparatus Company

Man Years (computed for the 12 month period)

Total: 0.7
Professional: 0.7
Other: None

Project Description:

Objective: We wish to apply a new Ultrasonic Flowmeter system to the solution of a variety of blood-flow problems.

Method Employed: A description of the new system is contained in the previous Individual Project Report NHI-200. During the past year the electronic circuitry has been redesigned and rebuilt.

Major Findings: By the use of better electronic circuitry, it has been possible to reduce the zero drift to less than 2% of a full scale flow of 200 cc/min. The device has been applied to measurement of blood flow in the artificial lung designed by Dr. Kolobow. On this application it has been possible to measure flow through the lung continuously for periods exceeding two days without appreciable technical difficulty.

Significance to Bio-Medical Research and the Program of the Institute:

Accurate, dependable measurement of blood flow continues to be a major problem. The ultrasonic flow transducer is inherently simpler and less trouble-prone than the magnetic flow transducer. Because the carrier frequency is much higher, ultrasonic transducers lend themselves more readily to telemetering through the skin, so that chronic implanting of transducers is facilitated.

Proposed Course: Several electronic schemes for determining the zero flow point without occlusion of the vessel have been proposed and will be investigated. Telemetry through the skin is promising and will be tested first in

mock-up form. In collaboration with Dr. Chris Stockbridge of Bell Laboratories and Dr. Tibor Foldvari of Harvard Apparatus Company we are transistorizing the electronics and converting the equipment to a form suitable for commercial fabrication.

Honors and Awards: None

Publications:

The following paper has been accepted for publication in the Review of Scientific Instruments:

1) Noble, F. W.: Dual - Frequency Ultrasonic Fluid Flowmeter.

Serial No. NHI - 380
Lab. of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Fast Phase Fluorometer

Previous Serial Number: None

Principal Investigator: Frank W. Noble

Other Investigators: Dr. Raymond Chen

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total: 0.3
Professional: 0.3
Other: None

Project Description:

Objective: The light impulse fluorometer of TRW is useful for the study of fluorescent lifetimes exceeding about 5 nanoseconds. Considerably shorter lifetimes may be measured by the use of phase shift fluorometry at high frequencies.

Method Employed: It can be shown that the behavior of a fluorescent substance when excited by sinusoidally modulated light is analogous to the performance of a simple resistance-capacitance low-pass filter in which the RC product is equivalent to the fluorescent lifetime, T. The phase delay of the fluorescent light with respect to the exciting light is

$$\phi = \tan^{-1} 2\pi fT$$

where f = light modulation frequency

Hence, if a measurement is made of the phase angle vs. the frequency, the lifetime T can be obtained. In order to measure small lifetimes, it is clear that the frequency should be large and the phase meter should be able to measure small angles.

Major Findings: A survey of commercial electro-optical modulators reveals that suitably modified stock units will modulate UV light down to 200 n.m. at frequencies exceeding 100 MHz. An analysis of the mechanism of the modulator shows that if an appropriate optical system is used and if the modulating voltage does not exceed half of the "half-wave" voltage for the modulator, the light exiting from the modulator consists of the sum of a steady component and a sinusoidal compenent at twice the frequency of the

driving voltage, plus small harmonic components. Hence, it is possible to reduce electrical cross-talk between the modulator driver and the optical receiver by the use of simple filters. This "second harmonic" system was first tested with a driving frequency of 60 Hz and with the unfiltered optical receiver connected to an oscilloscope. As predicted, the output from the receiver was a virtually pure 120 Hz sinusoid. Later the same system was tested with a driving frequency of 3.5 MHz and with the optical receiver tuned to 7 MHz. Again the results were as expected.

Significance to Biomedical Research and the Program of the Institute:

If the development is successful, the equipment will extend the measurement of fluorescent lifetimes to values much shorter than can be found with commercial apparatus.

Proposed Course:

Arrangements have been made with Isomet Corporation to supply a special modulator for test purposes. When this unit is received and if it operates as predicted, we plan to incorporate it in some form of highly sensitive second harmonic type of phase fluorometer, the exact design of which is still in progress.

Honors and Awards: None

Publications: None

Serial No. NHI -381
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of Sonication on Peripheral Blood Lymphocytes and Studies on the Mechanism of Lymphocyte Transformation.

Previous Serial Number: None

Principal Investigator: Arthur Turk

Other Investigators: P. R. Glade and L. N. Chessin

Cooperating Units: Laboratory of Clinical Investigations, NIAID

Man Years: (computed for the 12 month period)

Total: 0.3
Professional: 0.3

Project Description:

Progress During Past Year:

Transformation of peripheral blood lymphocytes to blast-like forms under the influence of various stimuli has far reaching implications, both in clinical medicine and investigation of the delayed hypersensitivity response. Although much data on the effects of stimulation is available, there is almost no literature on the mechanism of the phenomenon. Several lines of reasoning led to the hypothesis that the basic mechanism of transformation may be related to cellular injury. To test this hypothesis, the effects of sublethal sonication on short term lymphocyte cultures were determined and compared to effects of phytohemagglutinin in similar cultures. Cells stimulated by sonication or PHA showed similar blastoid alteration, similar subtleties of trypan blue staining during the first three days of culture and similar lysosomal patterns in the altered cells. However, cells from sonicated cultures did not display DNA or RNA synthesis rates above those seen in control cultures. In addition, trypan blue staining characteristics indicated a temporarily reversible injury in PHA stimulated cells, but not in sonicated cells.

The concept has been developed and supported that both PHA and sonication induced blast like transformation is related to cell damage.

Direction of Current Research:

This project is now completed.

Honors and Awards: None

Publications: None

Serial No. NHI-382
Laboratory of Technical Development
National Heart Institute

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Establishment of a Predictive Formula for Total Water Loss During Dialysis

Previous Serial Number: None

Principal Investigators: Arthur Turk, Robert L. Dedrick

Other Investigators: E. A. Gambos, J. Rosenthal, I. Siegel, J. Cooke

Cooperating Units: V. A. Hospital, Hemodialysis Unit, Washington, D. C.; NIAMD Artificial Kidney Program, BEIB, DRS

Man Years: (computed for the 12 month period)

Total: 0.05
Professional: 0.05

Project Description:

Progress During Past Year:

The total amount of water removed from the patient during hemodialysis is one of the most important dialysis parameters to carefully control. To establish a predictive formula with which the rate of water loss could be estimated, seven uremic patients undergoing chronic hemodialysis with a Kiil dialyzer utilizing cuprophane membranes were studied. Weight loss rate ranged from 0-9 gm/min. A Brookline bed scale, sensitive to $\pm$ 1 gram was used to measure weight change. Transmembrane hydrostatic pressure was assessed utilizing mercury manometers at the inlet and outlet sides of blood and dialysate lines. Osmolarity of blood and dialysate were measured in a freezing point depression apparatus and estimated independently by calculating the osmotic pressure contributions of electrolytes. Studies were made at blood flow rates of 70-338 ml/min., each patient being studied at several different flow rates.

Results indicated that blood flow rate in the ranges studied had no appreciable effect on ultrafiltration rate. For periods up to 12 hours, protein deposition on the membranes did not significantly alter the rate of ultrafiltration. Osmolarity difference between blood and dialysate in these chronically dialyzed patients by freezing point depression or direct calculation were in the range of 30-50 mosm/liter.

A least squares fit of the data for rate of weight loss versus transmembrane hydrostatic pressure difference produced the following predictive formula:

$dw/dt = .035 \Delta P - 0.98$

where dw/dt = weight loss in gm/min.

ΔP = transmembrane hydrostatic pressure difference in mm Hg.

At $dw/dt = 0$ the intercept of $P = 31$ mm Hg represents the hydrostatic head needed to offset the osmotic pressure difference tending to move water from dialysate to patient.

Use of the predictive formula in dialyses of five uremic patients produced four excellent agreements between predicted and actual weight loss and one poor agreement.

Direction of current research: The work has been completed.

Honors and Awards: None

Publications: None

971

Serial No. NHI -383
Lab. of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Exterpulsation Cardiac Assistance Device

Previous Serial Number: None

Principal Investigator: Arthur Turk

Other Investigators: None

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total: 0.1
Professional: 0.1

Project Description:

Progress During Past Year:

A complete counterpulsation system was assembled, including electronic synchronization between EKG and pressure application. A series of experiments in which normal dogs were counterpulsated were carried out. Results indicated that the magnitude of the effect on diastolic pressure and peak systolic pressure was small with the double layered polyvinyl chloride cuffs used to apply pressure over both dog legs from thigh to ankle. Pharmacologic interventions with Inderal, Mecholyl, acetyl-choline, Nembutal, and Levophed singly, and in various combinations to affect special autonomic states did not significantly improve the desired cardiac work lowering effects of counterpulsation. Because the shape and blood content of a dog leg is less desirable for producing the counterpulsation effect than a human leg, the results of dog experiments do not indicate that the technique will not work on human patients. Several cuffs, sized to dimensions of the human leg were fabricated, but it was considered unwise to expose humans to the risks that would be required for an adequately instrumented evaluation without further animal experiments.

Direction of Current Research:

There is no work currently being done on this project. The electronic synchronizer and cuffs are available for use if suitable clinical material becomes available.

Honors and Awards: None

Publications: None

Serial No. NHI -384
Lab. of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Long Term Perfusion of Animal Organs

Previous Serial Number: None

Principal Investigators: Arthur Turk and Robert C. Dutton

Other Investigators: None

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total: 0.8
Professional: 0.8

Project Description:

Progress During Past Year:

This project was undertaken to develop a method of perfusing isolated organs on a long term basis. Canine kidneys were used in the experimental system. A plan of incremental externalization was adopted; i. e., each step necessary for removal of the kidney from its normal environment would be individually and carefully evaluated. As a first step, the effect of renal artery catheterization on the otherwise undisturbed kidney was studied. A surgical technique to place a catheter into the renal artery from a carotid cutdown was developed. It was immediately apparent that the problem of thrombogenicity of foreign surfaces exposed to flowing blood had to be overcome. Because of the necessary flexibility of the renal artery catheter, a GBH surface was not applicable and large doses of anticoagulants were necessary to prevent severe renal embolism and infarction. Heparin dosages in the range of 15 to 20 mg per kg per day were necessary to provide the necessary level of anticoagulation. This dose is considerable higher than that used to provide a "therapeutic" level (clotting times averaging about twice control). Because the clotting times of blood from the animals in these experiments were so far above control, anticoagulants were given on a routine schedule without assessing the clotting before each dose. Function of the perfused kidney as assessed by urine specific gravity, urine volume and electrolytes and creatinine clearance, were near normal for as long as five days with the renal artery catheter in place and blood flow provided from the carotid artery.

Further surgical techniques were developed and a system of perfusion of an autologous extracorporeal kidney via an arterial catheter from the donor animal was established. Perfusion experiments up to 14 hours in duration established that the technique was workable and that several of the current perfusion problems, e. g., increased venous pressure, had been successfully solved. The single most important point in the perfusion system was found to be venous pressure control. If at any time during removal or perfusion, the venous pressure was allowed to sharply increase the kidney did not remain viable. Our technique allowed for removal and attachment of the kidney to perfusion apparatus in less than 90 seconds. The kidney was floated in a chamber of blood overlaid with silicone oil with the vein uncatherized. In this way, the venous pressure could be maintained at only a few cm of water and could be changed at will.

Function in the kidney perfused in this way was good. Arterial blood flow was easily maintained at 100 to 150 cc per minute, well above the necessary flow required for survival. The creatinine clearance of the perfused kidney dropped during the initial perfusion period but stabilized generally in less than an hour. The specific gravity and electrolyte composition of the urine from the experimental kidney was somewhat different than that of the contralateral control kidney but did indicate that the perfused organ was functioning at an acceptable level. Experiments could not be carried out for as long a time as desired because of severe hemorrhage in the bed of the removed kidney caused by the high levels of anticoagulation necessary to prevent catheter thrombosis. Scrupulous surgical technique did not ameliorate this problem because of excessive oozing, and we found it impossible to perfuse the kidney on a long term basis with autologous blood. Attempts to perfuse the kidney with isologous blood, so that the kidney donor would not have to be used as a perfusor, led to multiple petechiae and hemorrhages in the kidney with ultimate failure.

Direction of Current Research:

Continuation of this project in the manner originally outlined would have necessitated experiments on genetically matched dogs so that a kidney removed from one animal could be perfused by the blood of its genetic mate without damage to the perfused kidney. Although a source for matched animals was discovered, it was felt that there was not adequate personnel to further pursue the problem at this time.

Honors and Awards: None

Publications: None

Serial No. NHI-385
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Lymphocyte Culture in Agar

Previous Serial Number: None

Principal Investigator: Arthur Turk

Other Investigators: P. R. Glade and L. N. Chessin

Cooperating Units: Laboratory of Clinical Investigations, NIAID

Man Years: (computed for the 12 month period)

Total:0.3
Professional: 0.3

Project Description:

Progress During Past Year:

The in vitro study of lymphocytes has been hampered because current techniques require utilization of large numbers of cells and do not allow for evaluation of single cell responses. The fate of individual lymphocytes in tissue culture, and the possible role of cell to cell contact, can be directly analyzed by suspending the cells in agar before stimulation with antigen or mitogen. By this technique, the lymphocyte response may be measured by direct observation of the individual cells. The inherent possibilities of selective cloning and isolation of individual cells after stimulation by microdissection techniques are also apparent.

Leucocytes, 10^4 to 10^6 per ml, were suspended on 0.5 to 1.0% agar containing nutritive medium and sealed in glass capillary tubes, inside diameter 0.5 mm; many survived and continued to metabolize for 3 to 7 days while single cells were observed and photographed. By a technique similar to that used by Bowman in an antibiotic sensitivity assay, an agar suspension of mitogen or antigen, for stimulating transformation, may be deposited in the capillary lumen before introduction of the agar-cell suspension. In this way, the separated leucocytes are fixed before being exposed to mitogen or antigen, and are restrained from contact with other cells. Hence the isolated single cell may be observed and studied. Serial observation of cells in this system, using phytohemagglutinin, showed morphological changes characteristic of transformation at 48 and 72 hrs.

975

Studies of supravital dye uptake, however, indicated that cells in the center of the tube were less viable than those at the ends suggesting that limitation of CO_2 and O_2 exchange was responsible for the cellular injury.

Single cells can be grown in more favorable circumstances in a rectangular cross sectional container constructed of slides and cover slips. In this system, the entire surface of the agar may be exposed to the atmosphere in the incubator and gas exchange is not limited. This configuration is also more favorable optically because the lens effect of capillary tube curvature is avoided and a shorter working distance suitable for an oil immersion lens is obtained. Single cells were thus observed serially and photographed under high magnification phase contrast. Viability of cells so cultured was considerably better as assessed by supravital staining as compared to the capillary system. Mixed cell cultures can be studied in such a system without direct contact of the two cell populations by introducing each population in agar separately.

Direction of Current Research:

Methods of maintaining optimal growth conditions and determining viability of an isolated cell are being investigated. When the single cell in agar can be definitely identified as viable without sacrificing the culture, the method will be extended to investigation and photographing of single cell responses under a wide variety of stimuli.

The technique of lymphocyte culture in agar may also be extended for use in a system to automate the tedious direct cell counting necessary in lymphocyte transformation experiments. The Bowman bacteria counting apparatus will be employed for counting of transformed cells on the basis of size increase. For this procedure, cells may be suspended in the capillary tubes as described. The agar casts may then be expressed and grown in an open dish of culture medium until the time to assess transformation rate. Then, the casts can be replaced in the capillary tubes and counted in the apparatus. This technique will allow for optimal cell growth conditions but retain the capillary tube configuration which is now the basis for the bacteria counter.

Honors and Awards: None

Publications: Coulson, A. S., Turk, A., Glade, P. R. and Chessin, L. N.: Lymphocyte Culture in Agar. Lancet., 1:89, 1968.

Serial No. NHI - 386
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Title: Capillary Technique for Immunology

Previous Serial Number: None

Principal Investigator: Gerald G. Vurek

Other Investigators: E. Bruce Merchant

Cooperating Units: Laboratory of Immunology, NIMD

Man Years: (computed for the 12 month period)

Total: 0.2
Professional: 0.2
Other: None

Project Description:

Spleen cells activity producing antibody can be domenstrated by mixing them with erythrocytes in a gel matrix. In the presence of complements, the antibody causes lysis of the erythrocytes adjacent to the spleen cells, and clear zones around the spleen cells appear. These zones or plaques, can be counted and related to the conditions of sensitization, etc. Automatic or semi-automatic counting of the plaques is extremely desirable to obtain good statistics with reduced error.

Progress During the Past Year:

Preliminary results indicated the feasibility of adding cells, agar, and complement together to eliminate the two step procedure used in the conventioanl technique. The plaques were usually less than the diameter of the 1.5 mm capillaries used in the microbiological system, so that we tried several sizes to find the most appropriate. Tubes with inside diameter of 0.10 mm and 0.40 mm outside diameter were chosen as a result of a compromise between plaque number (small size meant small volume and therefore few plaques) and plaque size. Plaques were easily detected by light transmission measurements in contrast to the light scattering technique used in the bacteria counter.

A simple tube translating apparatus, light source, and photodetector were assembled as a prototype plaque counter. A synchronous clock motor pulls the tubes past a 50 μm hole which collimates the light from an incandescant lamp. The transmitted light is detected by a phototransistor. When a plaque appears in the light path, the transmitted light increases; the duration of the signal increase is related to the plaque size.

Counts of plaques by machine have agreed within 25% of the counts obtained by hand, using the conventional petri dish preparation. Plaque size distributions are also in reasonable agreement, although the capillaries have one dimensional geometry compared to the three dimensional geometry of the petri dish method.

Direction of Current Research:

We are continuing to evaluate the capillary system for its use in extending the application of the Jerne technique to immunochemistry. The dynamics of the cell populations producing antibodies can be studied easily with the Jerne technique and the capillary system will facilitate the performance of a variety of experiments.

Honors and Awards: None

Publications: None

Serial No. NHI - 387
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Capillary Techniques for Microbiology

Previous Serial Number: NHI-191

Principal Investigator: Gerald G. Vurek

Other Investigators: Viola M. Young, Harry H. Marsh III, R. L. Bowman

Cooperating Units: Infectious Diseases Section, Clinical Pathology, Clinical Center

Man Years: (computed for the 12 month period)

Total: 1.6
Professional: 0.8
Other: 0.8

Project Description:

Viable bacteria suspended in agar and nutrients inside a capillary will produce microcolonies which can be detected and counted by their light scattering properties. This report describes the progress of evaluating the capillary technique for rapid antibiotic sensitivity testing and total viable population in urine samples with commerical prototype instruments based on work originating in this laboratory.

Progress During Past Year:

Earlier work had shown that the addition of antibiotic to the molten-agar-test-organism suspension before its introduction into the capillaries provided a means of testing the antibiotic against that organism with the scanner. However, this procedure required a substantial amount of hand effort, was subject to the chance of cross contamination, and was generally inconvenient for rapid routine antibiotic susceptibility testing. We devoted some effort to the problem of precoating the capillaries with antibiotic which would be dry and stable. Coating the inside with liquids was not suitable; surface tension effects caused the coating to become non-uniform before it could dry. We were able to produce coatings which did not vary by more than 15 per cent of the mean over the length by using agar as a substrate. The antibiotic, at the level needed to produce the desired concentration in the filled capillary (usually between 0.39 and 100 μg/ml by steps of two-fold increase) was mixed with melted agar and was injected into Supramid tubing. Lengths of agar filled Supramid were put into capillaries and withdrawn by a pair of pressure rollers. As the Supramid was pulled and squeezed, the

979

antibiotic loaded agar was left in the capillaries as a "worm" along one side. Bundles of 30 to 50 capillaries were prepared at a time and were dried with a stream of tank nitrogen. Drying was complete within 20 minutes. These capillaries were usually stored in dessicant-containing glass tubes at -20°C. Stability tests indicate a useful life of more than 4 weeks. With an adequate supply of prefilled tubes assured, we were able to test the suitability of the capillary technique for antibiotic susceptibility testing.

With the assistance of the Infectious Diseases Section of the Clinical Pathology Department, we compared the antibiotic susceptabilities of 46 organisms as measured by the capillary method and by the standard tube dilution test method. The organisms tested included Gram negative and positive species, strict aerobes and facultative aerobes, and anaerobes. We used 14 of the commonly used antibiotics in the test.

The general procedure was to prepare racks of twenty tubes, including 9 levels of an antibiotic and a positive control in duplicate. The tubes were filled with agar and organisms, prepared from a water dilution of an overnight culture, and put in a 37°C incubator. Readings were made at 4,7, and 23 hours after the tubes were filled. The results, recorded as either growth or no growth, were compared with the overnight readings of the tube dilution tests. Both visual inspection of the capillaries and machine interpretation were used to evaluate growth. Pseudomonas aerugenosa required oxygenation of the medium, by bubbling before addition of the organisms, in order to get adequate growth for these tests. The time between addition of the organisms to the hot agar (50°C) and the time the capillaries were filled was usually less than 90 seconds. Approximately one hour was needed to fill twenty racks of tubes. Disposable sterile tuberculin syringes and needles were used to transfer the oranisms, mix them with the agar, and fill the capillaries. No other special precautions were taken to insure sterile preparation, the feeling being that the tests organisms concentration, 10^5/ml to 10^6/ml, would be far greater than stray contaminants. This was born out, as only a few tubes of the thousands prepared had noticable contaminants; these were determined to be yeasts.

We used the prototype scanner made by the CEC Division of Bell and Howell, Pasadena, California, for the mechanical scanner. This machine could scan a tube in less then ten seconds and print the count obtained. As with almost all prototype insturments, some mechanical and electrical difficulties were encountered, so that many of the tests were performed without its aid. In addition, the machine required a dedicated operator's complete attention for over one and one half hours during a 400 tube run, three times each day. We have recommended to the comapny that they devote more effort to make their equipment more reliable, more automatic, and faster, if their system is to be attractive as a part of an automated bacteriology laboratory. The American Instrument Company's first prototype also had similar problems.

We had to raise the sensitivity of the scanner to the point where a significant initial count was obtained in order to detect the slowly growing organisms. Fluctuations in the scanning speed produced variability in the

count, and complicated the interpretation of the counts. Hand analysis of analog records of the scattering signals indicated that growth indication could be made more reliable with the addition of perhaps a dozen channels of pulse-height analysis. This would allow us to follow departures from background more easily.

Of the 651 tests performed, nearly 70% showed agreement within one tube dilution between the capillary technique and tube dilution method. There seemed to be no systematic difference between the capillary technique and the standard method. In particular, tests with Neomycin, reportedly affected by anaerobic conditions, did not show any significant difference. We could not detect visible growth of these organisms reliably within seven hours. We could measure growth of all the organisms tested, including slowly growing Pseudomonas and Staphylococcus, within five hours, using the CEC apparatus.

Direction of Current Research:

Preliminary work has shown the value of pulse-height analysis and we are pursuing that line with commerically available equipment. In addition, it is clear that to follow the growth of individual colonies, we need a precise scanning system, with constant speed drive, reproducible tube positioning, and adequate electronics and optics. The first prototype of the American Instrument Company's version is being modified for this purpose.

Much of the difficulty is detecting a single bacterium arises from the diffuse scatter of the agar gel. The field of view of a single photodetector is several thousand times larger than a bacterium. We propose to try to subdivide the field into several parts and use single solid state photodetectors for each part. By using a circuit to select the largest signal from the group of detectors, it should be possible to improve the signal from bacteria with relation to the background gel.

The capillary technique may have applications to hematology in addition to the microbiological applications previously described. It may be possible to do erythrocyte and white blood cell counts, and perhaps distinguish platelets as well. Some preliminary studies will be made, including staining properties for differential counts.

Honors and Awards: None

Publications:

1. Bowman, R. L., Blume, P., Vurek, G. G.: Capillary Scanner for Mechanized Microbiology. Science 158: 78-83, 1967.

951

Serial No. NHI -388
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Colorimeter for Submicroliter Samples

Previous Serial No.: None

Prinicpal Investigator: Gerald G. Vurek

Other Investigators: R. L. Bowman, M. Burg

Cooperating Units: LKEM, NHI

Man Years: (computed for the 12 month period)

Total: 0.2
Professional: 0.2

Project Description:

Previous microcolorimeters have used capillary tubes with replaceable windows; the smallest volume meeded to fill the tube was usually greater than 2 μl. Spectrophotemetry of cells, parts of cells, and droplets ranging in volume down to 10^{-12}l has been performed with complex photometers using microscope optics. There is a need for a single colorimeter to handle samples down to 10 nl and having an optical path of 1 cm. This would permit detection of 10^{-13} moles or lesa of strongly colored materials.

Progress during the past year:

We have used "fiber optic" type glass fibers to carry light into and out of capillary cuvettes. These fibres are made of high index of refraction glass coated with an outer layer of low index of refraction glass. This construction insures total internal reflection of light passing down the fiber. The square cut end of the fiber will accept light with an effective numerical aperture greater than 0.5 so that the fiber can collect a great deal of light from a source.

The system we have built consists of the following units: a light source, a filter to select the proper wavelength for the material of interest, a condensing lens, the input fiber, the cuvette, the output fiber, the photo detector, and the read-out system. The material we have chosen to work with initially is chlorphenol, a compound which absorbs yellow light at alkaline-pH's. A three-watt incandescant lamp provides adequate light. A 10 nm band pass interference filter provides adequate wavelength discrimination for sample absorbances up to 0.8, corresponding to 15 μM. The condensing lens was a simple polymethyl methacrylate sphere"focussed" on the end of a 88 μm

982

outside diameter fiber 5 mm long. This fiber was inserted into the bore of a 0.4 mm O.D. by 0.10 mm I.D. by 12 mm long capillary. One end of the capillary was flared to guide the entrance of the fiber and provide a convenient filling "funnel". The output fiber also entered the bore of the capillary during measurement and carried the light to a 4473 side window photomultiplier. An adjustable dynode supply was used to provide sensitivity control; anode current was measured to obtain sample absorbance.

To fill the cuvette, a transfer pipette was placed at the flared end of the cuvette with the entrance fiber removed but the exit fiber in place. Capillarity of the clean cuvette caused it to fill completely. The entrance fiber was inserted to a preset position and the transmission was measured. At the completion of the measurement, both fibers were disengaged from the cuvette and it was cleaned by suction and water rinsing. In order to insure proper filling, the cuvette had to be dried completely; residual water films rapidly bridged the diameter of the tube and impeded filling. A few seconds of room air suction was adequate to dry the tube.

There was no difference in the linearity of the absorbance-concentration curve between that measured on a Beckman DU and the microcolorimeter over the range of zero to ten micromolar chlorphenol. Samples did not show effects of evaporation until one minute after the cuvette was filled and plugged with the fibers. Precision was better than one per cent including errors of pipetting and fiber placement. The sample volume needed for this unit was about 100 nl. The colorimeter, excluding readout and power supplies, was mounted on a 15 cm diameter disc.

Direction of Current Research:

We expect to try to reduce the sample volume requirement to 10 nl by using 30 μm fibers and capillaries. In addition, there exist solid state light sources emitting most of their energy in a narrow band in the red part of the spectrum. These, coupled with available sensitive solid state photodetectors would make an extremely compact special purpose unit. A simple spectrophotometer can be made using a multi-layer interference wedge as an adjustable monochrometer. Quartz fibers can be used for ultraviolet measurements. By mounting the phototube at right angles to the cuvette, the unit can be made into a simple fluorometer or spectrofluorometer. By attaching side arms to the cuvette, a flow system can be constructed which would be useful for routine microcolorimetry.

Honors and Awards: None

Publications: None

Serial No. NHI -389
Lab. of Technical Development
Bethesda, Maryland 20014

PHS - NIH
INDIVIDUAL PROJECT REPORT
July 1, 1967 through June 30, 1968

Project Title: Computer Simulation

Previous Serial Number: NHI-209

Principal Investigator: Bohdan Balko

Other Investigators: R. L. Berger, R. Moore, N. Davids

Cooperating Units: Pennsylvania State Univ., Department of Engineering Mechanics; Blood Program, National Heart Institute

Man Years (computed for the 12 month period)

Total: 1
Professional: 1
Other: 0

Project Description:

This is a continuation of work described in the PHS-NIH Individual Report for fiscal year 1966, Serial NHI-209.

The simulation technique referred to in the publications as finite element analysis has been applied to two types of problems. In the first case the physical principles are fairly well understood and the difficulty arises in the solution of the basic equations describing the system. In the other cases treated the system is not fully understood in terms of basic equations and is so complicated that only a description in terms of macroscopic parameters is possible. A method called compartmental analysis was applied to this type of problem.

In the first class belong the previously described thermal conduction and molecular diffusion problems. The method has been very successfully applied to these problems. We have now extended the method to mechanical problems such as simple, damped and complex oscillating systems and particle motion problems, i. e., the body problem. A correction scheme applied at each step of the simulation makes this approach two orders of magnitude faster than the earlier technique applied to similar problems to the same accuracy. We have also applied the method to quantum systems dealing with potential well and scattering problems. The development of these techniques for the analysis of physical systems should be especially helpful in the attempt to understand extremely complex biological systems on the microscopic scale.

The analysis of instruments by this technique has been initiated with the application of the method to output correction of a limited bandpass thermal amplifier.

The compartmental analyses technique was applied in the study of RBC. membrance activity. Extension was made to nucleated cells and to compartments simulating plasma membrane around cells. A series of simulated experiments were done with these and other experimental conditions of interest. All the above described programs were written in basic.

Direction of current research:

The application of the existing programs to various systems of interest will be continued.

Differential thermal calorimeter experiments will be conducted to obtain data for the R.B.C. membrane computer model.

Honors and Awards: None

Publications:

Balko, B., Berger, R. L.: Measurement and computation of thermojunction response times in the submillisecond range. Review of Scientific Instruments, Vol. 39, April, 1968.

Serial No. NHI-390
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Development of High Speed Mixers

Previous Serial Number: NHI - 206

Principal Investigator: Bohdan Balko

Other Investigators: Howard Chapman, Robert L. Berger

Cooperating Units: None

Man Years (computed for the 12 month period)

Total: 0.5
Professional: 0.25
Other: 0.25

Progress Description:

Progress During Past Year:

This is a continuation of work described in the PHS-NIH Individual Report for fiscal year 1966, Serial NHI-206. The 4 jet ball mixer has been used continuously in the high speed stopped-flow apparatus and is now available commercially.

A 4 jet-ball mixer, one half the size of the one used in the high speed flow apparatus has been made and utilized in a flow calorimeter. As expected from the theoretical analysis and experience with the larger model,excellent flow and mixing characteristics were observed.

Direction of Current Research:

The termination of the project is anticipated with the completion of the theoretical analysis of the mixer.

Honors and Awards: None

Publications: Berger, R. L., Balko, B., Borcherdt, W., Friauf, W.: High Speed Optical Stopped-Flow Apparatus. Review of Scientific Instruments, April 1968, Vol. 39.

Berger, R. L., Balko, B. and Chapman, H.: A High Resolution Mixer for the Study of the Kinetics of Rapid Reactions in Solution. Review of Scientific Instruments, April,1968,Vol.39.