ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL INSTITUTES OF HEALTH

1959

NATIONAL INSTITUTE OF
ARTHRITIS AND METABOLIC DISEASES

NATIONAL INSTITUTES OF HEALTH
PUBLIC HEALTH SERVICE
U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

Report of program activities

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Metabolic functions of nutrients in experimental animals.

Principal Investigators: Drs. J. G. Bieri, M. K. Ogivy Fou, J. E. Williams, Jr., C. J. Pölland, and H. E. Reid

Other Investigators: Miss M. M. Cullen, Mr. A. A. Andersen, and Mrs. D. P. Anderson

Cooperating Units: Dr. G. M. Briggs, Research Training Branch, DRG
Dr. M. Potter, Laboratory of Biology, NCI
Dr. R. E. Coggeshall, Basic Research, NINH
Dr. H. A. Barker, Department of Agricultural Biochemistry, University of California, Berkeley, California
Dr. H. H. Weissbach, Laboratory of Clinical Biochemistry, NHI

Man Years (calendar year 1959):
Total: 8
Professional: 4
Other: 4-1/3

Project Description:

Objectives: To determine the nutritional, biochemical, and physiological role of essential nutrients for experimental animals. To define the metabolic function of certain nutrients and to study interrelationships among these nutrients.

Methods Employed: Rats, mice, chicks, and guinea pigs are fed specially prepared, highly purified diets that contain adequate amounts of each nutrient known to be required by the particular species. The effects of specific deficiencies and imbalances are assessed by measurement of physiological, chemical, and enzymological changes in the animal, its tissues, and excreta. In cooperation with members of other laboratories, the relationship of nutrition to other scientific areas, such as neurology and pathology, is studied.

Major Findings:

A. Metabolism and function of fat-soluble vitamins.

1. Vitamin A. Vitamin A alcohol is esterified directly with free fatty acids by acetone powder preparation secured from pancreas. This system is relatively nonspecific with respect to fatty acids and may represent an additional mechanism for the esterification of vitamin A since evidence from other workers suggests an acyl coenzyme A dependent esterification in certain other tissues.

2. Vitamin E. Previous work attempting to describe the lesions seen in vitamin E deficient animals has been complicated by a simultaneous deficiency of vitamin A. For this reason, special dietary precautions were taken to assure uncomplicated deficiencies of each vitamin. The brains of vitamin A deficient chicks showed scattered pyknotic neurons most frequently in the optic tectum and Purkinje cell layer of the cerebellum. In vitamin E deficiency, large necrotic areas are seen in the cerebellum and occasionally in other parts of the brain. In the combined vitamin A and E deficiencies, many acellular areas occur, especially in the frontal lobe.

A long-term study of female chicks fed a purified diet containing selenium and a low level of unsaturated fatty acids, but no vitamin E or other antioxidants, was concluded. The vitamin E deficient chicks develop exactly like the supplemented controls. Following artificial insemination, fertile eggs are produced by both groups. The presence of tocopherol cannot be detected in tissues from deficient chicks. These results are regarded as evidence that vitamin E has no specific metabolic function.

Young chicks deficient in vitamin E but supplemented with selenium exhibit altered serum protein electrophoretic patterns concomitant with the appearance of exudative diathesis. Although the albumin/globulin ratio drops, there is little change in total serum protein. Factors other than changes in serum protein must be involved in the precipitous occurrence of the edema. A few chicks recover spontaneously; however, they show marked disturbances of the serum protein patterns.

Further study on the effect of certain organic solvents on respiratory enzymes indicates that the solvents combine with the enzyme to cause disruption of electron transport. The protective effects of vitamin E and other lipids are considered to be due to removal of the solvent from the enzyme rather than to a cofactor function. Preparations of cytochrome reductases that are made inactive by aging can be reactivated by the addition of tocopherol or any of several hydrocarbon compounds. It is thought that inhibitors form during aging which can be destroyed by the hydrocarbon lipids. These findings support the view that tocopherol does not participate directly in electron transport.

In a study of metabolic interrelationships between selenium, cystine, and tocopherol, it was found that dietary selenium and cystine exert an antioxidant action in certain tissues of the chick. This observation agrees with conclusions above that the exclusive biochemical action of vitamin E is that of an antioxidant.

B. Metabolism and function of vitamin B_{12} and folic acid.

1. Vitamin B_{12}. Research has continued with the vitamin B_{12} deficiency in chicks achieved by the use of a diet borderline in methionine and high in fat. Vitamin B_{12} has no effect upon the composition of the chicks' carcasses with respect to protein, fat, ash, and moisture. In contrast to the results with diets borderline in methionine, chicks fed a diet marginal in arginine do not show the same dramatic growth failure in the absence of vitamin B_{12}, when the fat content of the diet is raised. Apparently, there is a specific vitamin B_{12}-methionine metabolic relationship in chicks that is sensitive to elevated fat intake.

Vitamin B_{12} deficiency causes the chick to excrete large amounts of formiminoglutamic acid (FIGLU) in the urine which is similar to the finding in rats. Supplementation with vitamin B_{12} causes the excretion of this histidine metabolite to return to normal low levels after several days. A dietary supplement of methionine for one day causes an immediate drop in FIGLU excretion; then follows an increased rate of excretion above that of the pre-supplementation level. The excretion of FIGLU is very useful in establishing the metabolic interrelationships between vitamin B_{12}, methionine, and fat.

The growth-promoting activity of two light-sensitive analogues of vitamin B_{12}, described in Wolkstack, Barker, and others have been compared with vitamin B_{12} in chicks (in cooperation with Mrs. Wolkstack and Barker). These analogues, which are thought to be coenzyme forms of the vitamin, are benzimidazole-cobamide (BC) coenzyme and dimethylbenzimidazole-cobamide (DMBC) coenzyme; the latter should be the coenzyme form in animal tissues. The BC coenzyme promotes growth almost as well as vitamin B_{12} which is better than the 1/2 activity of the parent compound, aquomethyl vitamin B_{12}. The growth-promoting activity and the potency in restoring FIGLU excretion to normal of the DMBC coenzyme is equivalent to that of vitamin B_{12}.

2. Folic acid. Good growth can be restored to folic acid deficient mice by the dietary addition of procaine penicillin G. Both the procaine and the penicillin moieties had activity; the effect of the latter was probably mediated by the intestinal flora. Procaine, which is structurally similar to para-aminobenzoic acid, is probably converted to this compound in the body. In replacing dietary folic acid, procaine and para-aminobenzoic acid have equal activity, about one-tenth that of pteroylglutamic acid. Mice that were made severely deficient by dietary omission of folic acid plus 0.5% sulfasuxidine in the diet,

were injected with leukemia cells (in cooperation with Dr. Potter). With either a normal strain or a "folic acid antagonist-resistant" strain of cells, death is delayed considerably over that of control mice receiving folic acid. These results lend support to earlier data to indicate that metabolically a folic acid deficiency and the effects of administering a folic acid antagonist are quite different.

C. Dietary protein intake and maintenance of enzyme function.

1. Protein deficiency. The effect of protein deprivation upon the complete succinic oxidase system and its individual components in rat liver is being investigated. Preliminary results, when based on liver wet weight, indicate that after thirty days a protein deficiency produces only a slight decrease in the initial enzyme succinic dehydrogenase, no change in the cytochrome-b-cytochrome-c_1 complex, a 70% loss of cytochrome oxidase, a slight loss in succinate-cytochrome-c reductase, and a 90% loss of activity of the whole succinic oxidase system. With the exception of succinic oxidase, cytochrome-c, and cytochrome oxidase, new methods were devised for measurement of each component.

D. Highly purified diets and unidentified factors.

1. Purified diets. A highly purified diet in which protein has been completely replaced by amino acids was developed for the guinea pig. This diet supports normal growth and development, at a rate equal to that obtained with a diet containing well balanced protein. It is expected that this diet will be particularly useful in determining the essentiality of trace elements that may be present as contaminants in purified proteins.

2. Unidentified factors. The addition of various natural materials to a synthetic diet containing adequate amounts of all nutrients known to be required by the guinea pig causes an improvement in growth rate. When guinea pigs only three to five days old are fed the diet containing a source of the factor for two weeks their weight averages about 25 to 50 grams higher than that of the controls. This effect can be produced by a wide variety of natural materials--vegetable, cereal, or meat products. The most constant and richest source is alfalfa meal. Fractionation aimed at isolation of the active principle is planned.

Significance to NIAMD Research: A more complete understanding of the nutrition, biochemistry, and metabolism of essential amino acids, proteins, vitamins, minerals, fatty acids, and unidentified factors in different living organisms can be expected to contribute still further to our knowledge of the roles of these essential nutrients in human beings. It is well established that the nutrition of man plays a [illegible]

in the etiology of many degenerative and metabolic diseases, certain infectious and neurological diseases, and some types of cancer. Basic studies in nutrition and biochemistry of nutrients may provide the means to prevent or cure some of these diseases.

Proposed Course of Project: Efforts will be further directed along the lines of seeking the specific biochemical mechanisms responsible for the changes observed in the whole animal.

Part I included: Yes

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Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Agranoff, B. W., and Fox, M. R. Spivey. Antagonism of choline and inositol. Nature 183: 1259-1260, 1959.

Bieri, J. G. An effect of selenium and cystine on lipide peroxidation in tissues deficient in vitamin E. Nature 184: 1148-1149, 1959.

Bieri, J. G., Briggs, G. M., Pollard, C. J., and Fox, M. R. Spivey. Vitamin E metabolism in the chick. I. The normal growth and development of female chickens for extended periods without vitamin E or other antioxidants. J. Nutrition (in press).

Bieri, J. G., and Pollard, C. J. Serum protein changes in vitamin E deficient chicks. J. Nutrition 69: 301-305, 1959.

Briggs, G. M. Unidentified substances. In "Food, The Yearbook of Agriculture" 162-167, 1959.

Briggs, G. M. Nutrition and disease: Folic acid studies in the mouse. Am. J. Clin. Nutrition 7: 390-396, 1959.

Coggeshall, R. E., and Bieri, J. G. Pathology of the brain in simple and mixed deficiencies of vitamins A and E in the chick. J. Nutrition (in press).

Fox, M. R. Spivey, and Briggs, G. M. Effect of dietary lactone upon chicks fed a purified diet. Poultry Sci. 38: 964-968, 1959.

Fox, M. R. Spivey, and Nickelsen, O. Salt mixtures for purified-type diets. I. Effect of salts in accelerating oxidative rancidity. J. Nutrition 67: 123-136, 1959.

Fox, M. R. Spivey, and Nickelsen, O. Salt mixtures for purified-type diets. II. Effect of salts on the Maillard browning reaction. J. Nutrition 68: 289-296, 1959.

Fox, M. R. Spivey, Ortiz, L. O., and Briggs, G. M. The effect of dietary fat on vitamin B12-methionine interrelationships. J. Nutrition 68: 371-381, 1959.

Pollard, C. J., and Bieri, J. G. The destruction of vitamin A by blood. Brit. J. Nutrition 12: 359-366, 1958.

Pollard, C. J., and Bieri, J. G. On the occurrence of vitamin A aldehyde in fish and frog ova. Biochim. Biophys. Acta 31: 558-559, 1959.

Pollard, C. J., and Bieri, J. G. Further observations on the effect of isooctane on respiratory enzymes. J. Biol. Chem. 234: 1907-1911, 1959.

Pollard, C. J., and Bieri, J. G. Studies on the biological function of vitamin E. I. Tocopherol and reduced diphosphopyridine nucleotide-cytochrome c reductase. Biochem. Biophys. Acta 34: 420-430, 1959.

Pollard, C. J., and Bieri, J. G. Esterification of vitamin A by acetone powder from pancreas. Arch. Biochem. Biophys. (in press).

Pollard, C. J., and Bieri, J. G. Studies of the biological function of vitamin E. II. The nature of the specific activating effect of tocopherol in aged preparations of cytochrome reductases. J. Biol. Chem. (in press).

Reid, M. E. Guinea pig nutrition. Proc. Animal Care Panel 8: 23-33, 1958.

Reid, M. E., and Martin, M. G. Nutritional studies with the guinea pig. V. Effects of deficiency of fat or unsaturated fatty acids. J. Nutrition 67: 611-622, 1959.

Honors and Awards relating to this project:

Dr. John G. Bieri accepted a Fullbright Award which covers traveling expenses to Copenhagen, Denmark, to work with Prof. Henrik Dam, Nobel prize winner and world authority in the biochemistry and nutrition of vitamins in the Department of Biochemistry and Nutrition, Polytechnic Institute.

Serial No. NIAMD - 2
1. Nutrition & Endocrinology
2. Nutrition
3. Bethesda

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Individual Project Report
Calendar Year 1959

Part A.

Project Title: Diabetes--Effect of hormones on metabolism of fat and carbohydrate.

Principal Investigators: Drs. R. O. Scow and S. S. Chernick

Other Investigators: None

Cooperating Units: Dr. M. Rodbell, Laboratory of Cellular Physiology and Metabolism, NHI

Man Years (calendar year 1959):
Total: 5-1/3
Professional: 2
Other: 3-1/3

Project Description:

Objectives: To determine the influence of hormonal and other factors on the metabolism of fat and carbohydrate in normal and diabetic animals.

Methods Employed: Experimental animals deprived of one or more endocrine glands are treated with various hormones. The effects of extirpation and hormonal administration are studied in vivo and in vitro using conventional and isotopic techniques.

Major Findings: The study of the hormones involved in the development of diabetic ketosis has been continued. Last year it was reported that in pancreatectomized rats deprived of the pituitary or the adrenals the only hormone needed for the development of ketosis when insulin was withheld was a glucocorticoid. Growth hormone had no effect and ACTH was ketogenic only if the adrenal glands were intact. In subsequent experiments, dexamethasone has been used as the glucocorticoid because it produces the same effects as cortisone and only one thousandth as much is needed. The minimal effective dose of dexamethasone in a 150 gm. rat is 1 µg. per day.

The ketogenic action of dexamethasone is seen immediately after injection in insulin deficient rats hypophysectomized for one to two hours. However the ketogenic effect is delayed, at least

24 hours, in animals hypophysectomized for over a week and maintained with insulin and tube-feeding up to 17 hours before giving dexamethasone. If growth hormone is given with dexamethasone to the latter animals ketosis develops at once; growth hormone alone has no effect. In diabetics hypophysectomized for one to two hours, growth hormone given alone or with small doses of dexamethasone (0.5 µg.) has no appreciable effect on ketone body formation. Administration of insulin with 2.5 µg. of dexamethasone delays the onset of ketosis for at least seven hours. If growth hormone is also given ketosis develops. Several observations seem to indicate that growth hormone acts as a ketogenic agent by inhibiting insulin activity but this action occurs only when the insulin content is low. Measurements of the rate of ketogenesis in liver slices and of the liver fat content in the above animals suggests that the action of glucocorticoids and the anti-insulin activity of growth hormone, in the production of ketosis, occurs primarily in adipose tissue. Experiments are being designed to test this hypothesis.

The rate of ketogenesis in livers of diabetic and normal rats, studied with slices and with the perfused organ, has been found to be closely related to the amount of nonphospholipid fat in the liver. For a given fat content there is no difference in the rate of ketogenesis between the normal and diabetic rat. The rate of ketogenesis was the same whether it was determined on liver slices or in the perfused organ. Fatty livers of pancreatectomized rats, perfused for at least three hours, make 1.6 mg. of ketone bodies (measured as acetone) per gram of liver each hour. Addition of regular insulin, 36 units in 60 ml. of perfusate, had no effect on ketone body formation or on the glucose content of the perfusate. The perfusate at the end of the runs had at least 50% of the insulin added.

The metabolism of fat by liver is being studied in the perfused organ. The findings to date, using radioactive tripalmitin, indicate that triglycerides are readily taken up and incorporated into phospholipids, ketone bodies, and carbon dioxide. There is evidence that radioactive fat other than tripalmitin is being secreted into the blood.

The effect of insulin and its lack on utilization of ketone bodies has been studied in diabetic rats having a very low rate of endogenous ketogenesis. The rats were depleted of body fat by prolonged fasting prior to pancreatectomy. Utilization was studied by measuring the disappearance of ketone bodies from the blood following a single intravenous injection of either D(-)-betahydroxybutyrate or acetoacetate. Utilization of betahydroxybutyrate is reduced 60% in insulin deficient rats and can be readily restored to normal by insulin administration. Acetoacetate utilization is also impaired in the diabetic rat but to

a lesser degree than that of betahydroxybutyrate. These findings are in contrast to the generally accepted view that insulin has no effect on ketone body utilization. The studies in the pancreatectomized rat show that hyperketonemia, or ketosis, in insulin deficiency is the result of both increased production and decreased utilization of ketone bodies.

The fasting ketosis of pregnancy in rats also appears to be the result of an insulin lack in the tissues. The ketosis, which develops only during the last three days of pregnancy, is readily corrected by small amounts of insulin or Orimase. These rats are very sensitive to insulin and Orimase. Doses of Orimase which had very little effect on the blood sugar of nonpregnant animals produced severe hypoglycemia and death in the pregnant rats. It would seem that the high priority of the fetuses for glucose causes the blood glucose concentration in the fasting mother to remain at a level too low to stimulate secretion of insulin. Small amounts of glucose quickly correct the ketosis.

Last year it was reported that glucocorticoids and growth hormone, given singly, were not ketogenic in hypophysectomized fasting pregnant rats. It has now been observed that when the hormones are given together severe ketosis immediately develops. These findings are in agreement with those in the hypophysectomized-pancreatectomized rat given very small amounts of insulin.

Deposition and retention of radiocalcium and fluoride in the bone of growing rats was studied with the collaboration of Drs. R. C. Likins and I. Zipkin of the NIAMD. By inserting a steel pin into the tibia and taking serial roentgenograms, it was possible at the end of the experimental period to divide the tibia into several segments for analyses. One of the most important and interesting findings of this study was that much of the Ca^{45} and F released during remodeling of the ends of the growing bone did not enter the general circulation but was deposited in the immediate vicinity.

Significance to NIAMD Research: The pronounced alterations of fat metabolism seen in diabetes mellitus may be responsible for the early onset of vascular degeneration associated with this disease. The nature of the disturbances in lipid metabolism is poorly understood. The chief activity of this project has been directed to the study of early effects of insulin deficiency on fat metabolism, namely, mobilization of fat from adipose tissue to the liver, its conversion to ketone bodies and their utilization by peripheral tissues. The primary hormones involved have been more clearly defined. Studies using isolated organs and tissues are in progress to elucidate the site and mode of action of these hormones.

Proposed Course of Project: The study of hepatic metabolism of fat in the perfused organ employing radioisotopic techniques will be extended to determine the intracellular site of metabolism of

triglycerides and unesterified fatty acids, the sequence of events leading to their incorporation into ketone bodies and the nature of the fat being secreted into the blood (perfusate). At an early date, the effect of insulin and 'adipokinetic' hormones on these phenomena will be investigated. A method for perfusing isolated adipose tissue is being developed. This technique will be used to study the effect of hormones on the uptake and the release of fat, namely unesterified fatty acids and triglycerides. In vivo experiments will be continued to determine the role of growth hormone in the disturbed metabolism of fat in the diabetic.

Part B included: Yes

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Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Chernick, S. S., and Scow, R. O. Early effects of "total" pancreatectomy on fat metabolism in the rat. Am. J. Physiol. 196: 125-131, 1959.

2. Scow, R. O., Chernick, S. S., and Guarco, B. A.: Ketogenic action of pituitary and adrenal hormones in pancreatectomized rats. Diabetes 8: 132-142, 1959.

3. Scow, R. O. Effect of growth hormone and thyroxine on growth and chemical composition of muscle, bone, and other tissues in thyroidectomized-hypophysectomized rats. Am. J. Physiol. 196: 859-865, 1959.

4. Likins, R. C., Scow, R. O., Zipkin, I., and Steere, A. C. Deposition and retention of fluoride and radiocalcium in the growing rat. Am. J. Physiol. 197: 75-80, 1959.

5. Scow, R. O. Fat metabolism in experimental diabetes. In "Progress in Clinical Endocrinology" edited by E. B. Astwood. Grune and Stratton, New York (in press).

6. Scow, R. O., and Chernick, S. S. Hormonal control of protein and fat metabolism in the pancreatectomized rat. Recent Progress in Hormone Research 16: , 1960. (This paper was presented at the 1959 Laurentian Hormone Conference.)

Honors and Awards relating to this project:

None

Serial No. NIAMD - 3
1. Nutrition & Endocrinology
2. Nutrition
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Studies on germ-free and obese animals.

Principal Investigators: Drs. F. S. Daft, B. E. Gustafsson, O. Mickelsen, Z. M. Tolgay, R. S. Yamamoto, and Mr. E. G. McDaniel

Other Investigators: None

Cooperating Units: Drs. L. Sokoloff and G. L. Laqueur, Laboratory of Pathology and Histochemistry, NIAMD
Drs. L. T. Kurland and S. H. Faro, Epidemiology Branch, NINDB

Man Years (calendar year 1959):
Total: 12
Professional: 4
Other: 8

Project Description:

Objectives: To determine the nutritional, biochemical, and physiological characteristics of germ-free animals; to study the physiological and biochemical changes associated with obesity in experimental animals; to study experimentally a condition (Minamata disease) associated with the ingestion of sea food from a special area in Japan; to study the nutritional requirements of rabbits.

Methods Employed: Germ-free rats and guinea pigs are maintained on diets of known composition in sterile tanks. Their growth, blood picture, and other physiological functions are studied at various times after they have been started on the special diets. Germ-free animals are inoculated with single strains of bacterium to determine the organisms responsible for various phenomena seen in conventional animals.

The urine from rats that had been made obese by feeding a high fat diet was examined for a variety of proteins. The influence of dietary alterations on the excretion of these proteins was studied.

Cats and chicks have been fed diets to which were added a number of samples of Japanese sea food. This sea food was secured from the bay around which the patients with Minamata disease lived. The brain and organs of the animals were examined histologically and some organs and diet samples were analyzed for mercury.

Three week old rabbits have been fed purified diets deficient in thiamine with and without a thiamine antagonist. Urine and fecal samples have been tested for thiamine throughout the lives of the animals and when the animals were sacrificed, thiamine analyses were carried out on brain, liver, cecal contents, and a number of other tissues.

Major Findings:

1. Germ-free animals. Preliminary results indicate that neither antibiotics nor vitamin C has any vitamin-sparing effect in germ-free rats suggesting that the action observed in the conventional animals is mediated through the flora in the gastrointestinal tract. When germ-free rats were taken out of the tank and contaminated, they did not show a pantothenic acid-sparing effect even though the ration was supplemented with either penicillin or vitamin C. Efforts are being made to determine whether the lack of response in the ex-germ-free animals was attributable to incomplete contamination of the rat with all of the bacteria normally found in the conventional rats' gastro-intestinal tract or whether there are physiological differences between the conventional and ex-germ-free rats.

Conventional guinea pigs which have never been nursed, grow poorly for the first few weeks of life when fed a "complete" ration that produces excellent growth in guinea pigs that have been left with their mothers for two or three days. Work is in progress to determine the factor or factors required by the unweaned guinea pig. Two litters of germ-free guinea pigs have been born in the sterile tanks. The first litter of two pigs died three weeks after birth following what appeared to be normal growth and development. Autopsy showed lesions suggestive of a vitamin K deficiency. The diet for the second litter of three pigs is being supplemented with a complete vitamin mixture.

A device has been perfected which prevents coprophagy in conventional rats. This consists of a light-weight plastic tube which fits over the tail of the rat and in which the feces are collected. When rats are fitted with this device, they develop a folic acid deficiency on diets that permits normal growth and blood pictures in rats that can consume their feces. The rats with these "tail cups" pass feces which appear to have a different bacteriological flora than rats not so fitted. The Lactobacilli count in the feces of the tail-cupped rats was only 0.1% of the number present before the tail-cup was applied. This finding suggests that the development of a vitamin deficiency in the tail-

cupped rats may be due to a change in the flora of the gastrointestinal tract. The original observations suggested that the deficiency was due to the inability of the rat to ingest its feces and that the vitamins synthesized in the gastrointestinal trace were utilized only after the feces were consumed.

Germ-free rats develop a vitamin K deficiency with extensive hemorrhages and 100% mortality within 30 days after being started on a diet deficient in that vitamin. Conventional rats fed the same diet show no signs of a deficiency. The germ-free vitamin K deficient rats could be cured in 24 hours by various vitamin K compounds or by inoculation of the gastrointestinal tract with a single species of bacterium which had been isolated from conventional rats. This observation suggests that vitamin K is made available to the conventional rat primarily by one strain of bacterium.

The survival time of vitamin K deficient germ-free rats was significantly shortened by increasing the fat content of the diet. It has been known for many years that a vitamin K deficiency can be produced in conventional rats by adding a variety of sulfonamides to the diet. It was assumed that the latter compounds were producing the deficiency by changing the intestinal flora. Work in this laboratory shows that sulfaquinoxaline when added to a vitamin K-free diet produces a hemorrhagic diathesis in germ-free rats in two weeks while the sulfonamide-free diet produces the same effect in four weeks. The present evidence suggests that sulfaquinoxaline may act as a vitamin K antagonist.

Urinary calculi develop in 50% of the germ-free male rats which is associated with the excretion of large amounts of oxalic acid. Other work has shown that a pyridoxine deficiency increases the excretion of oxalic acid. In the germ-free and in the tail-cupped conventional rats, a pyridoxine deficiency does not increase the incidence of urinary calculi.

2. Obesity. Serial carcass analyses of the rats on high fat diets show that these rats from the beginning of the dietary regimen have a higher fat content associated with their more rapid rate of growth. Outwardly, these animals appear to be similar to older rats of the same body weight maintained on the low fat diet. The increase in fat was found as early as five weeks on the diet.

The livers of the obese rats were larger than those in the lean controls but in spite of this, the concentration of water, protein, and fat were the same in the two groups. The concentration of total carbohydrates was higher in the livers of the obese rats. The kidneys and hearts in the obese rats were larger than those in the lean controls but the proximate composition was essentially the same in both.

The muscle (right gastrocnemius) of the obese rat contained slightly more fat than that of the lean animals. The weights of the adrenal glands in the obese rats were about 1.5 times those in the lean rats. The heavier adrenals had a higher concentration of fat (primarily neutral) and vitamin C. The concentration of phospholipid and cholesterol were the same in the two sets of glands.

The obese rats had a slower removal rate for intravenously injected glucose--resembling diabetics. The concentration of vitamin B_{12} in plasma and livers of obese rats was greater than that in lean controls even though dietary concentrations of this vitamin were the same. This was unexpected since other work in the laboratory suggests that the requirement for vitamin B_{12}, at least in the chick, is increased by a high fat diet. The absorption of an oral dose of vitamin B_{12} was greater in the obese than in the lean rats. The difference in absorption was not due entirely to the differences in fat content of the diets since adult rats that had been fed the high fat diet (which produced the obesity) for several weeks showed the same absorption of vitamin B_{12} as the lean rats.

A greater degree of proteinuria is seen in the older male obese rats than in the lean. Lean female rats excrete very little protein; the obese female rats do. The urinary proteins were mainly albumin and β-globulin which differs from other forms of proteinuria where all the serum proteins are present. The degree of proteinuria in both the obese and the lean rats was decreased by the presence in the diet of 15% of an inert filler (Solka-floc).

Although the Sprague-Dawley and NIH rats become obese when fed a high fat diet, a strain (S5B/N) produced by crossing the two does not become obese. The S5B/N rats can accurately regulate their calorie intake regardless of the fat content of the diet.

3. Rabbits. It has been reported that rabbits do not develop a thiamine deficiency presumably since they consume their "night" or soft feces which contain large amounts of vitamins. When three to four week old rabbits were placed on a thiamine-free diet, they gained weight at about the same rate as their supplemented controls but excreted considerably smaller amounts of the vitamin in their urine; the concentration of the vitamin in the feces and livers of the "deficient" group was less than that in the controls whereas the cecal contents had the same concentration of thiamine. So far, four out of eight rabbits maintained on the deficient diet for one to two hundred days developed ataxia whereas none on the supplemented diet did so. When a thiamine antagonist was given, the rabbits on the deficient diet showed greater weight losses and required a longer period of time for recovery than those on the supplemented diet.

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4. Minamata disease. When Dr. Leonard Kurland (NINDB) was in Japan a number of years ago, he investigated a localized epidemic of a peculiar neurological disease. Sea food from the adjoining bay was implicated as the cause since all victims were fishermen or ate sea food caught by a member of the family. Cats and birds in the area were reported to show the same condition. The sea food brought back by Dr. Kurland was fed to cats. They developed severe neurological disturbances associated with paralysis of the hind limbs and convulsive seizures. We have secured evidence that the toxicity of the sea food is considerably less on a horse meat regimen than on a vegetable stock-milk diet. Day old chicks are much more sensitive to the toxicity than cats. Future work will be done with the chicks.

The Japanese reported that a mercury catalyst in the effluent from a vinyl plastics plant on the edge of the bay was responsible for the symptoms. Although we get neurological symptoms in the animals fed organic mercury compounds, the course of the disease and the amount of mercury required to produce the symptoms are slightly different from that seen with the sea food.

Significance to NIAMD Research: The work with the germ-free animals will give us a better understanding of the method whereby animals utilize those vitamins which are made available to them by the flora in the intestinal tract. These animals are developing some interesting leads as to the factors that may be involved in blood coagulation in the intact animal.

Obesity is a major health problem in the United States. The obesity developed in the rats more closely resembles that in human beings since it is produced entirely by dietary means.

The studies on Minamata disease may be important for public health workers since at least a dozen plants in this country are now using the same process as the Japanese for manufacturing vinyl plastics.

Proposed Course of Project: Work on the obese rats will be continued in an effort to determine the changes in the composition of organs and tissues as the animals become obese. Further studies will be made on the factors that appear to influence proteinuria in the rat. The work with germ-free animals will probably be expanded during the coming year to other areas than the nutritional studies.

Studies will be carried out to determine whether the conditions produced in animals fed the Japanese sea food is due entirely to mercury.

Part B included: Yes

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Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Windmueller, H. G., Ackerman, C. J., Bakerman, H., and Mickelsen, O. Reaction of ethylene oxide with nicotinamide and nicotinic acid. J. Biol. Chem. 234: 889-894, 1959.

Mickelsen, O., and Anderson, A. A. A method for preparing intact animals for carcass analyses. J. Lab. Clin. Med. 53: 282-290, 1959.

Mickelsen, O. Water, In "Food, The Yearbook of Agriculture" 168-172, 1959.

Mickelsen, O. The effect of high calcium intakes--Introduction. Federation Proc. (in press).

Honors and Awards relating to this project:

Dr. Olaf Mickelsen was elected to the Editorial Board for The Journal of Nutrition for a three-year term.

Serial No. NIAMD - 4
1. Nutrition & Endocrinology
2. Nutrition
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Effect of dietary phosphates on dental caries in children.

Principal Investigators: Drs. I. I. Ship and O. Mickelsen

Other Investigators: Drs. F. J. McClure, R. C. Likins, I. Zipkin, A. L. Russell, and Mr. C. L. White, NIH
Dr. H. Schraer, The Pennsylvania State University
Drs. B. Beasley, G. E. Waterman, and Miss H. C. Olson, Division of Indian Health
Misses M. Talcott and B. Wenberg, South Dakota State College of Agriculture and Mechanic Arts

Cooperating Units: Division of Indian Health
Home Economics Department, South Dakota State College of Agriculture and Mechanic Arts
Department of Physics, The Pennsylvania State University
Bureau of Indian Affairs

Man Years (calendar year 1959):
From NIAMD:
Total: 1/3
Professional: 1/3
Other: 0

Project Description:

Objectives: To determine whether the addition of phosphates to the diets of children will reduce the incidence of dental caries; whether the addition of phosphates to the diets of children will effect the growth and development; to evaluate the safety of the phosphates and calcium added to the diets; and to evaluate the efficacy of added calcium (the other component of the phosphate compound) as a means of reducing the absorption and retention of strontium^{90}.

Serial No. NIAMD - 4

Methods Employed: Two per cent calcium phosphate has been added to bread in four boarding schools and the addition of a placebo (flour) to the bread in four control schools. Neither the subjects nor the investigators know the identity of the schools. Total number of children in the study is 1800. Routine examinations include an annual dental examination including bilateral bite-wing radiographs on all children, quarterly measurements of height and weight on all children, annual bone density determinations using hand radiographs on a sample of 200 children from both the control and supplemented schools, annual saliva collection and analysis on a sample of 200 children from both the control and supplemented schools, regular food intake surveys including collection and analysis of representative diets in all schools, controlled balance study on a small group of children in a control and a supplemented school for calcium, phosphate, magnesium, and strontium^{90}, annual physical exams for nutritional status on a sample of children in both control and supplemented schools, and annual blood urine collection on a sample of children in both groups of schools for hemoglobin (blood), calcium, phosphorus, and magnesium (urine).

The students in eight boarding schools in North and South Dakota are under the jurisdiction of the Bureau of Indian Affairs. There is a total group of 3600 children; those included in the study are all the seven to fourteen year olds (1800).

Major Findings: Preliminary dental survey shows a moderate incidence of caries and periodontal disease among these children. Arrangements are completed for the addition of the supplement and the placebo to the bread in all schools. This started with the beginning of school in September, 1959. The children in the supplemented schools will receive approximately one gram of added calcium per day. This, they will receive throughout the school year.

The Indian children on entering school (five to seven years of age) appear to be markedly underdeveloped in so far as height and weight are concerned. The older children compare favorably with the standards for white children.

Significance to NIAMD Research: Although fluorine added to drinking water reduces the incidence of dental caries by 50 to 65%, there is still a large increment of caries to be taken care of. There is a public health need to develop a means of reducing the incidence of dental caries among the people who do not have access to a fluoridated water supply. The addition of calcium offers an opportunity to evaluate the safety of long-term calcium supplements and its effects on growth and development. It offers a means of securing an answer to a fundamental public problem, namely; will added calcium reduce the absorption and retention of strontium^{90}.

Serial No. HIAMD - 4

Proposed Course of the Project: To arrange a two to three week balance study during the first part of the summer vacation (June, 1960) in selected control and supplemented schools. Repetition of balance studies will be carried out in June, 1961. To continue the investigation as above outlined for one and possibly two additional years.

Part B included: No

Annual Project Report
Calendar Year 1959

Serial No. NIAMD- 5
1. Nutrition and Endocrinology
2. Experimental Liver Diseases
3. Bethesda

Part A.

Project Title: Biochemistry and Physiological Role of Factor 3 and Other Selenium Compounds

Principal Investigator: Klaus Schwarz and Calvin M. Foltz

Other Investigator:

Cooperating Units: None

Man Years (calendar year 1959):
Total: 3-1/3
Professional: 1-1/3
Other: 2

Project Description:

Objectives: To study the biological effects of Factor 3 and other selenium compounds, to investigate their natural distribution, determine their chemical nature and their biological specificity. To differentiate and delineate Factor 3 deficiency diseases from other deficiencies, especially vitamin E deficiency. To isolate and characterize Factor 3, to devise methods for the chemical synthesis of Factor 3-active compounds, and to investigate their clinical effects.

Methods Employed: Biopotency of selenium compounds and Factor 3 is determined by animal assays, using protection against dietary liver necrosis in the rat as test system. A micro-analytical method, radio activation analysis, and a sensitive colorimetric assay are used for selenium determinations. Synthetic methods are applied for the preparation of various selenium compounds, especially of selenium-containing sulfur amino acid derivatives. Fractionation and isolation techniques (chromatography, counter-current distribution, etc.) are used for the further fractionation of Factor 3 preparations from natural sources, and also for the purification of other natural compounds.

Major Findings: The further systematic screening of synthetic selenium compounds was carried out in collaboration with Prof. Dr. Arne Fredga at the University of Uppsala, who synthesized "tailor made" organoselenium compounds for this study.

Part B included Yes ☑ No ☐

Tests of homologous series of aliphatic mono- and diseleno carboxylic acids have led to the establishment of certain rules determining Factor 3 activity. For instance, the monoseleno-dicarboxylic acids tested have been far less potent than their diselenium analogues. An optimum of biopotency was found when the selenium was in the γ position from a carboxyl group. All of the isomeric diseleno-dibutyric acids have been studied and none were found to be as active as γ,γ'-diseleno-di-n-butyric acid. Substitution of the selenium-carrying carbon atom with a methyl group further increased biopotency. The resulting diseleno-γ,γ'-di-n-valeric acid showed an ED_{50} of 1.4 γ per cent selenium, as compared to .7 γ per cent selenium for natural Factor 3. This valeric acid derivative is a racemic mixture. Attempts at resolution are under way in order to determine the biopotencies of the optically active forms. Also, a large quantity of the compound has been synthesized and put at our disposal, so that now toxicity studies and therapeutic trials can be carried out.

Tests of other organoselenium compounds again demonstrated the fact that, while many types of organoselenium compounds exhibit activity, minor changes in structure quite often produce profound changes in Factor 3-potency. The ED_{50}'s of a series of ten 2,1,3-benzoselenadiazoles, obtained from Dr. F. E. Ray of the Cancer Research Laboratory of the University of Florida, ranged from 4 μg to 40 μg of selenium.

The improved isolation scheme for Factor 3, employing mild conditions, has been refined further and most of the prepurified starting material has been processed by this method. The concentrate from this procedure is being used for further attempts to characterize the biologically active form of Factor 3 by means of such techniques as electrophoresis, paper chromatography, column chromatography and counter-current distribution. A method to determine selenium compounds on paper chromatograms by means of neutron activation of the paper as a whole is being developed in collaboration with the Oak Ridge National Laboratory. It is expected that gamma ray spectrometry of the activated chromatograms will permit the location of Factor 3-selenium. Results thus far have been inconclusive because of the presence of interfering radioactive isotopes, for example gold and iron, in the irradiated chromatograms.

It was discovered that sulfur amino acids have a profound sparing effect on the requirement for vitamin E. A thorough study of the effects of sulfur amino acid-supplementation on dietary liver necrosis showed that these compounds do not prevent liver necrosis by themselves; but they delay the onset of the disease. Sulfur amino acids free from biologically active traces of selenium, as established by radioactivation, were used. Supplementation of .5 per cent selenium-free L-cystine, or equivalent amounts of homocystine or DL-methionine, reduced the level of vitamin E required for the prevention of liver necrosis to 1/10th. While normally .73 mg per cent of dl-α-tocopheryl

acetate in the diet affords 50 per cent protection, 70 µg per cent suffice to produce the same effect in the presence of .5 per cent L-cystine or .62 per cent DL-methionine. Tests to investigate whether resorption sterility shows the same phenomenon are under way.

With selenite as liver protecting agent, the effect of sulfur amino acid addition is much less pronounced. The reduction of the ED_{50} for selenite amounts to about 50 to 70 per cent. These effects are only produced by those sulfur amino acids which are in the pathway of normal metabolism. Sulfite, sulfate, taurine, etc., were inactive. Combination of suboptimal levels of selenite and dl-α-tocopheryl acetate, on the other hand, showed that there was a slight mutual potentiation. For the establishment of this effect, a minimum of ca .3 mg per cent of vitamin E in the diet is necessary.

Significance to NIAMD Research: The studies have yielded results of basic significance for the understanding of biochemical and nutritional phenomena. Factor 3, a potent biochemical agent, is effective in several species in preventing fatal necrotic lesions of liver, heart, kidney, muscle, and other tissues. The discovery that Factor 3 is a selenium compound has opened up numerous questions of scientific interest and also has practical implications. It may lead to a significant contribution to the understanding and the treatment of necrotizing diseases in the human.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Schwarz, K., Stearns, J. P., and Foltz, C. M., Relation Between Selenium Traces in L-Cystine and Protection Against Dietary Liver Necrosis. Metabolism 8, 88 (1959).

2. Schwarz, K., Roginski, E. E., and Foltz, C. M., Ineffectiveness of Molybdenum, Cadmium and Cobalt in Dietary Necrotic Liver Degeneration. Nature 183, 472 (1959).

3. Schwarz, K., Der Faktor 3, das Selen, und die Ernährungsbedingte Nekrose. Vitalstoffe-Zivilisationskrankheiten IV, 1 (13)(1959).

Honors and Awards relating to this project:

None

Individual Project Report
Calendar Year 1959

Serial No. NIAMD- 6
1. Nutrition and Endocrinology
2. Experimental Liver Diseases
3. Bethesda

Part A:

Project Title: The Role of Vitamin E and Factor 3 in Metabolism and their Relation to Dietary Necrotic Liver Degeneration.

Principal Investigator: Laurence M. Corwin

Other Investigators: Klaus Schwarz

Cooperating Units: None

Man Years (calendar year 1959):
Total: 2-1/3
Professional: 1-1/3
Other: 1

Project Description:

Objectives: To elucidate the modes of action of vitamin E and Factor 3. To clarify the chain of events in the development of dietary liver necrosis and analogous diseases (heart muscle necrosis, muscular dystrophy, etc.).

Methods Employed: Mitochondria and microsomes were prepared by differential centrifugation according to the method of Schneider and Hogeboom. Oxidations were studied in a Warburg respirometer. Mitochondrial swelling was studied in the Beckman spectrophotometer and measured as the decrease in optical density at 520 mμ at room temperature. Phosphate was determined colorimetrically by the method of Fiske and Subbarrow. Radioactive measurements were carried out by means of a windowless Geiger-Muller tube counter.

Major Findings: Vitamin E-deficient mitochondria have higher succinate cytochrome c reductase and malate dehydrogenase activities than those from vitamin E supplemented animals, as reported previously. It was postulated that the deficient mitochondria may allow greater access of substrate to enzymes due to structural damage. In studies on the relation of hypotonicity and metabolic factors to swelling of deficient and supplemented mitochondria it was found that the former did swell more. In the presence of AMP, swelling was markedly reduced.

Part B included Yes ☑ No ☐

Under these conditions the -E and +E mitochondria behaved alike. It is possible that vitamin E may have an effect on metabolism which in turn effects the structural integrity of the mitochondria. Since the metabolic basis for mitochondrial swelling has been an object of controversy, studies to elucidate some of the major issues were undertaken. It was confirmed that oxidative phosphorylation protects mitochondria against swelling in a hypotonic medium, despite a known protective effect of 2,4-dinitrophenol (DNP). The latter was shown to prevent and accelerate swelling under different conditions. DNP protects against swelling in media wherein oxidative phosphorylation is not possible. In medium permitting phosphorylation DNP accelerates swelling when AMP is the acceptor. When ADP is the acceptor, however, protection occurs. None of the other agents uncoupling oxidative phosphorylation (dicumerol, azide, and thyroxine) has been found to possess all the effects of DNP on swelling. The protective action of DNP has been shown to be independent of its uncoupling effect. It has been hypothesized that its site of action may be adenylate kinase at the mitochondrial surface.

In attempts to explain the prevention of mitochondrial swelling by certain reducing agents, swelling was studied under conditions in which different respiratory enzymes were in an oxidized or reduced state. With methylene blue it was shown that reduction of the carriers was not necessary for protection by antimycin A and cyanide. Blocking of the oxidation of the succinate chain has little effect in the prevention of mitochondrial swelling, as compared to the effect obtained by inhibition of the electron transport chain of DPN-linked substrates. A synergistic action between ADP and the respiratory inhibitors is thought to be a key to the understanding of mitochondrial stability.

Simultaneous lack of vitamin E and Factor 3 in the diet results in respiratory decline, a defect which characterizes the latent phase of dietary necrotic liver degeneration. Liver slices of such rats are unable to maintain normal oxidation. However, when mitochondria are prepared from these livers, no such decline is observed with the various members of the tricarboxylic acid cycle as substrate, with the exception of succinate. With the latter, decline occurred upon addition of DPN to the medium; this phenomenon has been attributed to an accumulation of oxalacetate. It was detected that homogenates of livers during the latent phase of liver necrosis showed decline of respiration very similar to that observed in the slice if α-ketoglutarate or succinate were used as substrate. Dietary vitamin E prevented the decline of α-ketoglutarate oxidation fully, and that of succinate oxidation mostly. Dietary Factor 3 (as selenite) was without effect on these systems. In vitro supplementation of a physiological concentration of α-tocopherol (5 γ/50 mg tissue), completely prevented decline,

as did the antioxidant DPPD and a tocopherol metabolite (the Simon-Milhorat factor).

Since the mitochondria themselves do not exhibit decline, and the homogenates do, some other particle or factor in the homogenate must be combined with the mitochondria to elicit decline of respiration. The microsome fraction, but not the soluble supernatant fraction, was shown to have this property. Moreover the microsomes caused a marked lowering of the P/O ratio of mitochondria with α-ketoglutarate as substrate. In vitamin E-deficient homogenates and mitochondria P/O ratios were found to be lowered with α-ketoglutarate as substrate. Some component from the microsomal preparation is inhibitory to respiration and to oxidative phosphorylation. This agent is sensitive to boiling for one minute. It is apperently released in vitamin E-deficient homogenates. However, microsomal preparations from E-supplemented animals also cause this effect, particularly with ageing. The nature of this inhibitory agent is being investigated further.

Significance to NIAMD Research: An elucidation of some of the factors involved in mitochondrial stability is of significance in the study of many disease states involving cell structure. From the evidence presented it seems certain that vitamin E controls some metabolic mechanism which, in turn, are related to the maintenance of structural integrity. Clarification of the nature of this metabolic role of vitamin E should aid in a better understanding of its role in preventing dietary liver necrosis.

Proposed Course of Project: The nature of the agent in microsomes which appears to be responsible for the respiratory decline in homogenates, as well as the lowering of the P/O ratio of α-ketogluterate, will be studied further. Of maximum interest will be the metabolic circumstance which accounts for the instability of the microsomes in the vitamin E-deficient state. Based on electron microscopic evidence indicating the breakdown of microsomes and mitochondria in the latent phase of liver necrosis, the metabolic interrelationship of these cellular particles will be investigated closer. It is also planned to study the effects of dietary Factor 3 on these phenomena.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project

1. Corwin, L. M., and Schwarz, K., An Effect of Vitamin E on the Regulation of Succinate Oxidation in Rat Liver Mitochondria. J. Biol. Chem. 234, 191 (1959).

2. Corwin, L. M., Oxalacetic Decarboxylase from Rat Liver Mitochondria. J. Biol. Chem., 234, 1338 (1959).

3. Lipsett, M. N., and Corwin, L. M., Studies on Stability of Rat Liver Mitochondria: I. Role of Oxidative Phosphorylation in Swelling. J. Biol. Chem. 234, 2448 (1959).

4. Corwin, L. M., and Lipsett, M. N., Studies on Stability of Rat Liver Mitochondria: 2. Relation of the Electron Transport System to Swelling. J. Biol. Chem. 234, 2453 (1959).

Honors and Awards relating to this project:

None

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD- 7
1. Nutrition and Endocrinology
2. Experimental Liver Diseases
3. Bethesda

Part A:

Project Title: Biological Significance of the Glucose Tolerance Factor (Chromium(III)), and its Relation to Glucose Utilization and Diabetes.

Principal Investigator: Walter Mertz

Other Investigators: Klaus Schwarz

Cooperating Units: None

Man Years (calendar year 1959):
Total: 3-2/3
Professional: 1-1/3
Other: 2-1/3

Project Description:

Objectives: To investigate the effects of chromium(III)-deficiency in experimental animals, to study the mechanism by which GTF-active chromium(III) compounds improve glucose tolerance, to assay the effects of such compounds on glucose uptake in in vitro systems, to develop optimal ways of application of chromium(III) complexes, to study their effect on diabetes in experimental animals and in the human, to study the role of the liver on glucose utilization, and to elucidate the correlation between chromium(III) in blood and "insulin like" principles.

Methods Employed: Male rats are maintained on various natural and semi-synthetic diets, and glucose removal rates are measured after intravenous injection of 125 mg glucose per 100 g of weight. GTF potency is determined by comparison of glucose tolerance before and after application of a single dose of GTF by stomach tubing. GTF preparations are added to various GTF-deficient diets, and glucose removal rates are determined in rats, maintained on such diets for various periods of time.

Epidydimal fat tissue is removed from rats raised on a GTF-deficient diet, as well as on GTF-supplemented rations. Glucose uptake by this tissue is measured with and without GTF.

Chemical and physical fractionation procedures are applied to natural sources of GTF. Stability of GTF activity in various purified fractions is measured.

Major Findings: The glucose tolerance factor has been identified with chromium(III). This element is the active ingredient of the dietary factor which is necessary for the maintenance of normal glucose tolerance in the rat. The finding puts chromium on the list of trace elements required for well being and normal function of the animal organism, and possibly the human as well.

In the course of the purification procedure applied to natural sources of GTF, fractions had been obtained which cured the impairment of glucose tolerance in rats with a single dose of 50 to 100 µg per 100 g of weight. These purified fractions lost their activity when stored at +4°. Inactivation was accompanied by the precipitation of a fine, brownish material. Wet ashing of crude sources of GTF, as well as of purified preparations, on the other hand, did not destroy the activity. The finding indicated the involvement of a trace element as active component. Of all the elements, some were eliminated because of their ample occurrence in GTF-deficient diets, and others because of their properties. Salts of 47 elements were tested as to their GTF potency. Only trivalent chromium(III) was effective. Hexavalent chromium salts were found to be inactive.

Screening of a great number of chromium(III) compounds, all of which are coordination complexes, showed that very stable coordination compounds were inactive. Examples are the acetylacetonate or ethylenediamine complexes. They are inert and cannot be utilized. The oxalato, salicylato and bis-biguanide complexes, on the other hand, showed a high degree of potency. These compounds are more labile and can relinquish the chromium for utilization in the tissues. Only 20 µg of chromium(III) are required per 100 g rat weight to cure the glucose tolerance factor deficiency.

A method for the production of chromium(III) complexes, using chromic acid and sulfur dioxide, has been devised for the synthesis of coordination complexes. A large variety of chromium(III) coordination derivatives of amino acids, pyrimidines, purines, nucleosides and nucleotides, as well as of biologically occurring bases have been prepared and assayed in the glucose tolerance test, with the aim to find the most potent and therapeutically most suitable chromium(III) complex. The identification of naturally occurring complexes of chromium(III), for instance in serum, also has been initiated.

Measurements of glucose uptake showed that the isolated fat tissue of rats on a chromium(III)-supplemented diet removed almost twice as much glucose from the medium than that of controls on a GTF-deficient diet. For this effect, the presence of insulin is required.

The addition, in vitro, of chromium(III) compounds was found to increase glucose uptake of fat tissue in this system. A full effect was obtained with .1 µg per flask. As in the above experiment, small amounts of insulin were required. The increased glucose uptake could be accounted for by an increase of fat synthesis in the tissue. In experiments using radioactive glucose, chromium(III) supplementation enhanced the incorporation of labeled glucose carbon into fat approximately three fold.

Chromium(III) was found to be well tolerated. In contradistinction to sixvalent chromium, which has no GTF activity, it is relatively nontoxic. Dietary supplementation of various compounds of chromium(III) revealed no signs of toxicity when 1 mg per cent of the element was supplemented for 6 weeks. More than 1 g of the element per kg of weight was tolerated well by rats when it was given by stomach tube.

The effect of GTF-active chromium(III) complexes is being studied in alloxan diabetic animals. Rats with stabilized insulin requirement, food intake and blood sugar levels, two months after alloxan treatment responded to the application of chromium(III) by lowering of fasting blood sugar levels, which were brought down from 250 to 125 mg per cent and by ceasing urinary ketonbody excretion.

Significance to NIAMD Research: The reported findings indicate that chromium(III), even though previously considered of no importance for the animal organism, is essential and indispensable for the maintenance of normal carbohydrate metabolism. This finding may be highly significant for future research in diabetes and other diseases. Low intravenous glucose tolerance is the earliest and most sensitive symptom of incipient diabetes in the human. In animals on GTF-deficient diets the low glucose tolerance is the only symptom recognized thus far, but more severe manifestations may be expected on diets more rigidly free of GTF-active chromium(III). It is conceivable that chromium(III) may be a hitherto "missing link" in diabetes. The discovery of chromium(III) as the active principle of GTF may have opened up new experimental and therapeutic approaches to this disease.

Proposed Course of the Project: The site and mode of action of GTF-active chromium(III) compounds will be investigated. To this end, the various aspects of glucose metabolism and the effect of chromium(III) thereon will be studied, for instance in isolated fat and muscle tissue. Rigidly chromium(III)-free diets will be developed to observe the long-term effects of chromium(III) deficiency. The influence of chromium(III) supplementation on alloxan diabetic rats is being studied. These studies will be extended, and the results applied to human diabetes and associated disturbances. To elucidate the mechanisms by which chromium(III) is handled and maintained in physiological form, the assimilation of chromium(III) is to be investigated in yeasts and other microorganisms. Also, the special systems will be investigated through which the animal organism may absorb GTF-active chromium(III) and deal with it in the regulation of carbohydrate metabolism.

NIAMD- 7

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Mertz, W., and Schwarz, K., Relation of Glucose Tolerance Factor to Impaired Intravenous Glucose Tolerance of Rats on Stock Diets. Am. J. of Physiol. 196, 614 (1959).

2. Schwarz, K., Mertz, W., and Simon, E. J., In vitro Effect of Tocopherol Metabolites on Respiratory Decline in Dietary Necrotic Liver Degeneration. Biochim. Biophys. Acta 32, 484 (1959).

3. Schwarz, K., and Mertz, W., The Terminal Phase of Dietary Necrotic Liver Degeneration in the Rat. Metabolism 8, 79 (1959).

4. Mertz, W. and Schwarz, K., Prevention of Respiratory Decline in Necrotic Liver Degeneration by Antioxidants in vitro. Proc. Soc. Exp. Biol. and Med. (in press).

5. Schwarz, K., and Mertz, W., Chromium(III) and the Glucose Tolerance Factor. Arch. Biochem. and Biophysics 85, 292, (1959).

6. Schwarz, K., Der Glukose-Toleranz-Faktor, seine Identifizierung und physiologische Bedeutung. Congress Report - Deutsche Gesellschaft für physiologische Chemie. Berlin 1959.

Honors and Awards relating to this project:

None

PHS - NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD-8

1. Nutrition & Endocrinology
2. Endocrinology
3. Bethesda

Part A.

Project Title: Study of Anterior Pituitary Hormones: Isolation of Hormones.

Principal Investigators: Dr. Robert W. Bates and Dr. Peter G. Condliffe

Other Investigators: Mr. Tulane B. Howard, Mrs. Mary M. Garrison, Dr. Jacob Furth, Dr. Richard Fraps, Dr. A. Albert, Dr. Sidney Werner, Dr. Leonard Warren and Dr. Richard A. Miller.

Cooperating Units: Harvard University, USDA (Beltsville), Mayo Clinic, and Columbia University.

Man Years (Calendar Year 1959):
Total: 3 2/3
Professional: 1 2/3
Other: 2

Project Description:

Objectives: To isolate protein hormones, especially anterior pituitary hormones from glands; from functional, transplantable mouse or rat pituitary tumors, and from blood; to study the primary and secondary structure of protein hormones, especially the anterior pituitary hormones; to investigate the nature of these hormones as they circulate in the bloodstream, to correlate chemical structure and biological function.

Methods employed: Hormones are extracted from pituitary glands, rat or mouse pituitary tumors, and from blood. Crude extracts which may contain several hormones are fractionated by techniques commonly used in protein chemistry, such as salt precipitation, solvent fractionation, isoelectric precipitation, preparative electrophoresis, ion-exchange chromatography and counter-current distribution. Various quantitative biological assays are required to determine the concentration of the several hormones in the protein fractions. Physical properties of the purified hormones are determined by free electrophoresis, sedimentation, diffusion and other techniques for the study of proteins. Identification and quantitative

estimation of the amino acid composition of the purified hormones are made by paper and ion exchange chromatography. Kinetic methods for the study of organic reactions are used to prepare suitable derivatives of the hormones for structural analysis.

Major Findings: Studies on isolation of protein hormones from glandular tissue and blood have been expanded this year to include prolactin, gonadotrophins, and insulin, as well as the known hormones of the anterior pituitary.

Thyrotrophin (TSH). One reason for studying the transplantable TSH-producing pituitary tumors of mice was the hope that TSH uncontaminated with the other pituitary hormones would be found. The absence of the known hormones, growth hormone, prolactin, ACTH, FSH and LH, has been demonstrated. This year, Dr. Albert tested mouse tumor fractions for exophthalmogenic activity, using the increase in the intraocrnial distance in Fundulus. A negative response was found with a tumor extract, although the amount of TSH injected was 1,500 times greater than that in a bovine pituitary extract which produced a positive response. This clearly proves that TSH is not the exophthalmogenic-producing substance.

The separation of TSH and LH (luteinizing hormone) from pituitary extracts has never been satisfactorily accomplished by classical methods of fractionation. With the cooperation of Dr. Richard Fraps, who did the LH bioassays, it has been found that at pH 9 - 9.5 and a low salt concentration, TSH is adsorbed on a diethylaminoethyl cellulose (DEAE-C) column while LH is not adsorbed and passes through the column. This is another demonstration of the usefulness and efficiency of ion exchange adsorbents.

By a combination of ion exchange chromatography using DEAE-C and starch gel electrophoresis, it has been found that TSH activity is associated with several different proteins. This finding greatly complicates the problem of isolating pure TSH.

Extracts of TSH from human blood plasma were made for Dr. Sidney Werner who found the thyrotrophic effects of these fractions, as well as those from human pituitaries, to be neutralized by antiserum to bovine pituitary thyrotrophin. This indicates a lack of species specificity in the case of TSH.

Blood Plasma Extraction. Solubility studies have shown that the success of the "dry to wet" (percolation) procedure for the extraction of certain protein hormones from blood plasma depends upon the fact that most blood proteins are denatured in 75-80% ethanol-saline, so that they are no longer soluble in more aqueous-ethanolic media at pH 7. Only part of the plasma proteins are denatured at lower concentrations of ethanol.

Insulin from Blood: By using I^{131} labeled insulin, it has been found that the original "dry to wet" extraction procedure can be simplified. It is not necessary to lyophilize the blood plasma. Instead, an amount of NaCl is added to the plasma, before 4 volumes of 95% ethanol are added, such that the final concentration is 2% NaCl and 76% ethanol. Upon standing at room temperature for 30 minutes, the plasma protein is denatured and the usual low weight yield of 2-4% of the total plasma proteins is extracted with about 80% of the insulin.

Prolactin: A transplantable pituitary tumor of rats, so-called mammotropic tumor, obtained from Dr. Jacob Furth, is being grown and studied for its hormone content. Preliminary studies show the tumor to contain a concentration of prolactin, growth hormone, and ACTH, that is only 1% of that of the pituitary. But, because the tumor is several hundred times the size of the pituitary, the female rats have adrenals 10 times normal size, greatly enlarged mammary glands distended with milk, and infantile nipples, showing a lack of gonadotrophic stimulation of the gonads.

Sialic Acid: In collaboration with Dr. Leonard Warren, it has been found that, with increasing dosage of TSH, the depletion of sialic acid from the thyroid glands of baby chicks occurs in parallel with the depletion of stable iodine and I^{131}. Sialic acid is a constituent of thyroglobulin, the storage form of the thyroid hormone in the thyroid gland.

Significance to NIAMD research: The mechanism of action of pituitary hormones is not, as yet, understood. Progress will depend upon availability of pure hormones, a knowledge of their structure, and the accuracy and simplicity of the bioassay procedures. Progress in this area has been achieved by our improved methods for isolation and bioassay of the hormones. It is hoped that these methods will permit determination of blood levels of TSH in patients.

Proposed Course of Project: TSH is one of the few pituitary hormones which has not been isolated in pure form. Efforts to prepare pure TSH from various raw materials will continue and, when successful, full chemical and physical characterization will follow. The physico-chemical homogeneity of TSH and prolactin will be assessed. Amino acid composition will be determined, together with the terminal amino acids. For this purpose kinetic studies on enzymatic degradation and on the preparation of chemical derivatives will be carried out. Studies on the effect of TSH on thyroid physiology in the chick will continue. Time course studies will be made on the blood levels of TSH in radiothyroidectomized mice implanted with TSH-producing tumors

to determine when the blood levels of TSH rise. Determinations of the TSH level in blood of patients will continue. Studies on blood content of prolactin and insulin are planned, using our new extraction procedure. A new micro method for detection and bioassay of prolactin is being sought.

Part B included.

Part B:

Honors, Awards, and Publications

Publications other than abstracts from this project:

Bates, R. W., Garrison, M. M., and Howard, T. B.
Extraction of thyrotropin from pituitary glands, mouse pituitary tumors and blood plasma by percolation. Endocrinology 65, 7-17, 1959.

Condliffe, P. G., Bates, R. W., and Fraps, R.
Fractionation of bovine TSH and LH on cellulose ion-exchange columns. Biochim. & Biophys. Acta, 34, 430-438, 1959.

Bates, R. W., Albert A., and Condliffe, P. G.
Absence of exophthalmogenic substance in transplantable TSH-producing tumors of the pituitary of mice. Endocrinology 65, 860-861, 1959.

Bates, R. W. and Condliffe, P. G.
Studies on the chemistry and bioassay of thyrotropins from bovine pituitaries, transplantable pituitary tumors of mice and blood plasma. Recent Progress in Hormone Research, Academic Press, New York, N. Y. (In press).

Condliffe, P. G., Bates, R. W., Garrison, M. M. and Howard, T. B. On the existence of multiple forms of bovine thyrotropin. Biochim. & Biophys. A. (In Press).

Honors and Awards relating to this project:

Dr. Peter G. Condliffe accepted a Fellowship from the National Foundation, which covers traveling expenses to Copenhagen, Denmark, and return, and salary for a year from September 1, 1959, to work with Dr. Ottesen in the Carlsberg Laboratories in Copenhagen.

Serial No. NIAMD - 9

1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda

Part A.

Project Title: Studies on the secretion and metabolism of adrenocortical steroids in man and animals.

Principal Investigator: Dr. Hildegard Wilson

Other Investigators: Dr. Lillian C. Butler (Georgetown)
Dr. Mortimer C. Lipsett (NCI)
Dr. Saul Rosen (NIAMD)
Dr. David W. Ryan

Cooperating Units: Georgetown University School of Medicine
National Cancer Institute
Clinical Endocrinology Branch, NIAMD - 1520

Man Years (Calendar Year 1959):

Total:	2.5
Professional:	1.5
Other:	1.0

Project description:

Objectives: (1) Clarification of the derangements in adrenocortical biosynthetic processes in adrenal carcinoma, (2) Validation and extension of the procedure for surveying urinary steroid patterns, (3) Cooperating with clinical endocrinology staff, NIAMD, and other investigators in (a) the diagnosis of endocrine patients and (b) specialized problems of steroid analysis.

Methods and Major Findings: (1) (a) Methods have been developed for the quantitative determination of 3 Δ^5 steroids in urine, viz. Δ^5-17αOH-pregnenolone (5-17-PG), its chief metabolite, Δ^5-pregnenetriol (5-PT) and dehydroepiandrosterone (DHA).

Three persons without adrenocortical abnormality excreted about 55 ug of 5-PT per day, while 2 others had none. Five patients with adrenocortical carcinoma excreted increased amounts up to 15 mg., while in 3 subjects with Cushing's syndrome (1 with adrenal carcinoma), 5-PT was absent. No 5-17 PG was found in any of the adrenal carcinoma urines, but all had elevated amounts of DHA (6-90 mg.)

The question of whether DHA originates from 5-17-PG in the adrenal cortex could not be answered by a study of the ratios of 5-PT and DHA in urine. The only correlation was that both were elevated in adrenal carcinoma. Our results do, however, suggest that 5-17 PG may not be a regularly synthesized precursor of DHA.

(b) High excretion levels of Δ^5 steroids were observed in adrenal carcinoma patients with relatively low levels for Δ^4-3-ketone metabolites. On this basis the suggestion that Δ^5 steroids appear only above a threshold when the 3β-ol-dehydrogenase system has been saturated is not tenable.

(2) (a) Urine extracts prepared from subjects who had received C^{14} cortisol were chromatographed on our partition column. That our procedure accurately separates the C_{21} metabolites of cortisol was shown by the recovery of 70% of the applied radioactivity in the "cortisol metabolite" fraction, and 30% in other fractions known to contain breakdown products of cortisol.

(b) Ancillary procedures for the determination of additional steroid metabolites have been developed and applied.

Pregnanediol; has been found to be a major metabolite in adrenal carcinoma, (27 mg. per day in one subject).

Pregnanetriol; formerly believed to be excreted only in small amounts in adrenal carcinoma, has been found greatly elevated (up to 20 mg. per day).

(3) Six patients from the Clinical Endocrinology wards have been studied. In all, the possibility of an adrenal tumor was ruled out by our analyses. Various other adrenocortical abnormalities were delineated.

Significance to NIAMD Research: The long range significance and objective of these studies is the elucidation of the general relations between adrenocortical activity and disease states. Understanding of the exaggerated situations found in adrenal tumor patients should aid in understanding normal processes.

A metabolite hitherto little studied, Δ^5-pregnenetriol, has been found characteristic of most cases of adrenocortical malignancy. Moreover, it was detected in the urine of normal persons as well. In general, our findings support the theory that DHA arises from Δ^5-17-OH-pregnenolone, the precursor of Δ^5-pregnenetriol, but the possibility of another source is not excluded.

Proposed Course of Projects:

(1) The study of the patterns of urinary steroids in adrenal carcinoma patients will continue as outlined.

(2) Further collaborative studies with the NIAMD Clinical Endocrinology staff, concerning blood plasma steroids in certain disorders are being initiated.

Part B included.

Part B:

Publications other than abstracts from this project:

Wilson, H., Lipsett, M. B., and Butler, L. C.
Steroid excretion in hypophysectomized women, and the initial effects of ACTH. A study in urinary steroid patterns.
J. Clin. Endocrin. & Metab. April, 1960.

Wilson, Hildegard
Steroid Hormone Metabolites: Their Origins, Distribution and Measurement. Proceedings of the Applied Seminar on Lipids and Steroid Hormones. Ed. F. William Sunderman, Lippincott (In Press).

Wilson, Hildegard
Some Principles of Partition Chromatography as Applied to Steroids. Proceedings of the Applied Seminar on Lipids and Steroid Hormones. Ed. F. William Sunderman, Lippincott (In Press).

Wilson, Hildegard
Column Chromatographic Methods for the Analysis of Neutral Urinary Steroid Metabolites. Proceedings of the Applied Seminar on Lipids and Steroid Hormones. Ed. F. William Sunderman, Lippincott (In Press).

MIN - PHS
Individual Project Report
Calendar Year 1959

Serial No. NIAMD- 1
1. Nutrition & Endocrinolog
2. Endocrinology
3. Bethesda

Part A.

Project Title: Neuroendocrine studies - Mesencephalic-hypothalamic mechanisms influencing the anterior pituitary.

Principal Investigators: Dr. Evelyn Andersen, Dr. H. Wilson.

Other Investigators: Dr. Spence (Georgetown), Dr. Kanta (WRAIR) Dr. Raymaker (AFIP), and Mr. Koger.

Cooperating Units: Georgetown University School of Medicine, Walter Reed Army Institute of Research, and Armed Forces Institute of Pathology.

Man Years: (Calendar Year 1959):

Total:	3
Professional:	2
Other:	1

Project description:

Objectives: To obtain further evidence for the hypothesis which this group has postulated, namely, that there is a mesencephalic hypothalamico-pituitary activating system closely linked to the reticular activating system of Magoun.

Methods employed: Destruction of areas in the midbrain in the dog by a neurosurgical approach and by electrolytic lesions in the midbrain of the cat by stereotaxic technique. ACTH release is measured by quantitative assay of urinary corticoids. Methods for the determination of corticoids in dog and cat urine have been developed in this laboratory. Catecholamines in the urine are determined by a modification of the von Euler method.

Major findings: In some preliminary studies on cats with lesions in the midbrain, there occurred as much as a 10 fold increase

over the normal in urinary adrenal steroids in response to stress, while in other cases no rise in steroid excretion following stress occurred. The anatomical studies locating the lesions are under way.

In a series of dogs it has been shown that transection of the upper midbrain disturbs the hypothalamico-pituitary activating system. In a number of dogs in which the operation was not successful, the opposite effect was noted, i. e., a very marked increase of corticoids in response to stress.

There is little or no change in the amount of catecholamines in the urine following transection of the midbrain in the dog or following electrolytic lesions in midbrain in the cat.

Significance to the program of NIAMD: Central nervous system influences on metabolic and endocrine activity are of tremendous importance in the study of human metabolic diseases.

Proposed course of project: A study is under way in which electrodes are to be placed in the brain of squirrel monkeys and cats. Urinary corticoids will be studied following electrical stimulation and later electrolytic lesions of areas in the midbrain and hypothalamus.

Part B not included.

NIH - 135
Individual Project Report
Calendar Year 1959

Serial No. NIAMD - 11
1. Nutrition & Endocrinology
2. Endocrinology
3. Bethesda

Part A.

Project Title: The influence of the central nervous system on metabolic functions - Creatine and creatinine metabolism.

Principal Investigator: Dr. Kathryn Knowlton

Other Investigator: Mr. Leonard H. Hedda

Man Years: (Calendar Year 1959)
Total: 1 1/3
Professional: 1
Other: 1/3

Project Description:

Objective: To distinguish between extrusion of intracellular creatine and failure of creatine incorporation by tissues as causes of creatinuria following transection of the spinal cord or other damage to the central nervous system.

Methods employed: The body creatine of a dog was labeled with N^{15} by feeding creatine synthesized to contain 60 atoms % excess N^{15} in the glycine moiety. The spinal cord was then transected at the C_7 level. From the urines collected starting at the time that the labeled creatine was fed, the excreted creatine and creatinine were isolated, degraded to sarcosine and purified as the toluenesulfonyl derivative. The N^{15} content of these products was then determined by mass spectrometry.

Major findings: Immediately after the spinal cord transection, creatine appeared in the urine in the large amounts usually seen after this operation. However, contrary to most of our experience the creatinuria ceased for about 10 days before reappearing. The N^{15} labeling of the second creatinuria was only a little lower than that of the concurrently excreted creatinine.

Since urinary creatinine is known to be derived immediately from the creatine of muscle, this approximate identity in N^{15} labeling

of urinary creatine and creatinine appears to establish muscle creatine as the source of most of the creatinuria following spinal cord transection. The consistent but rapidly changing course of the P^{15} labeling during the first creatinuria lends itself to interesting speculation, but not to easy evaluation.

Significance to NIAMD research: Since creatine phosphate occupies a place of some importance in intracellular energy interchanges of the mammalian body, knowledge of various phases of its metabolic mechanisms contributes to understanding of diseased states. The above finding gives a preliminary answer to one question among the many needed to explain the muscle atrophy and paralysis following isolation of peripheral tissue from the central nervous system centers above the level of spinal cord transection.

Proposed course of project: Plans are being made to repeat this study in an improved and more decisive experimental design after termination of my service. Such studies might be extended to the creatinuria following midbrain transection.

Part B not included.

PHS - NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD - 12
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda

Part A:

Project Title: CNS and Aldosterone Secretion

Principal Investigator: Dr. Evelyn Anderson

Other Investigators: Dr. James Davis (NHI), Dr. William T. Spence, and Dr. Webb Haymaker.

Cooperating Units: NHI, Georgetown University School of Medicine, and AFIP.

Man Years (Calendar Year 1959):
Total: 1
Professional: 1
Other:

Project Description:

Objectives: To investigate the claim of other workers that the midbrain and pineal body elaborate a neurohumor (glomerulotropin) which controls the secretion of aldosterone by the adrenal cortex.

Methods and Major Findings: Normal dogs secrete a measurable amount of aldosterone into the adrenal vein blood and in the urine. This is increased several fold when a ligature is placed on the vena cava. Both normal and caval dogs are subjected to a midbrain transection. Urinary aldosterone is measured before and after the operation. On the 2nd post-operative day the left adrenal vein is cannulated and the rate of aldosterone secretion is measured. The findings show that the rate of aldosterone secretion is not altered by the midbrain transection.

Significance to NIAMD Research: This should clear up erroneous conclusions which are being published in the current literature, that the midbrain and pineal gland elaborate a hormonal substance which stimulates the release of aldosterone.

Proposed Course of Project: At present the conditions which control aldosterone secretion are not understood. The problem will be pursued.

Part B not included.

PHS - NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD - 13
1. Nutrition & Endocrinology.
2. Endocrinology.
3. Bethesda.

Part A.

Project Title: ACTH Studies

Principal Investigators: Mrs. Frances Wherry, Dr. Evelyn Anderson, and Dr. Robert W. Bates.

Other Investigators: None

Man Years (Calendar Year 1959):
Total: 1
Professional: 2/3
Other: 1/3

Project Description:

Objectives: To investigate ACTH level in blood under various conditions.

Methods and Major Findings: The project so far has consisted of the development of an assay method for minute amounts of ACTH. The method is based on the measurement of corticosterone in adrenal venous blood following the intravenous injection of micro amounts of ACTH into hypophysectomized rats. The corticosterone is determined by a spectrophotofluorimetric method. The findings so far indicate that an amount of ACTH as low as 50 mU causes a significant rise in the corticosterone level of adrenal vein blood.

Methods for the extraction of ACTH from plasma are being worked out. It is anticipated that a reliable method for the determination of ACTH in blood can be worked out.

Significance to NIAMD research: This method should be of great value in studies of pituitary and adrenal abnormalities.

Proposed Course of Project: Studies will be carried out on the influence of the CNS in the release of ACTH into the blood stream.

Part B not included.

PHS - NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD - 14
1. Nutrition & Endocrinology
2. Endocrinology
3. Bethesda

Part A.

Project Title: Insulin Studies

Principal Investigators: Dr. Evelyn Anderson, Dr. Robert W. Bates and Mrs. Frances Wherry

Other Investigators: Dr. A. S. Renold and Dr. James B. Field

Cooperating Units: Harvard University and NIAMD (CE). 145C

Man Years: (Calendar Year 1959)
Total: 1 1/3
Professional: 2/3
Other: 2/3

Project Description:

Objectives: To investigate the factors influencing the rate of secretion of insulin.

Method: A method for the bioassay of micro amounts of insulin and a method of extraction of insulin from plasma have been developed. The level of insulin in the pancreatic vein blood of dogs has been measured during and immediately following the intravenous administration of one of the following: growth hormone, prolactin, ACTH, and tolbutamide. This has been compared with the insulin level following administration of saline and of 10% glucose.

Major Findings: Insulin content of peripheral blood of the dog during fasting is approximately 37 uU/ml. plasma; pancreatic blood: 60 uU/ml; following infusion of glucose 275 uU/ml. plasma. Following the administration of either growth hormone, prolactin, ACTH, or tolbutamide there is no rise in the level of insulin. The conclusion is that an elevated blood glucose is the only known stimulant to an increased rate of insulin secretion.

A collaborative study has been carried out with other laboratories at NIH and Harvard, comparing the assay of insulin in plasma by

in vitro and *in vivo* methods. Preliminary evidence suggests that there is poor agreement between the *in vitro* assay methods using the rat diaphragm and the fat body. Furthermore, neither the data using the rat diaphragm nor using the fat body agree with the *in vivo* method used in this laboratory.

Significance to NIAMD Research: It is very important to the study of diabetes to have reliable information on levels of insulin in blood in diabetic patients.

Proposed course of project: To continue the present study to clear up the confusion between the present methods of assay.

Part B not included.

PHS - NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD - 15
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda

Part A.

Project Title: Hypophyseal and extra hypophyseal control of activity in peripheral cells of the pigeon.

Principal Investigator: Dr. Richard A. Miller

Other Investigators: Dr. Robert W. Bates and Mrs. Mary M. Garrison

Man Years (Calendar Year 1959):
Total: 2/3
Professional: 1/3
Other: 1/3

Project description: (Part I)

Objectives: To determine the nature and source of the stimulus, evoked by insulin, formaldehyde, gonone, etc., which causes a hypertrophy of the adrenal in the pigeon after the removal of the pars distalis.

Methods employed: Insulin was injected for 4 to 7 days into pigeons from which (a) the pars distalis alone, or (b) the pars distalis and the infundibular process of the hypophysis, were removed. The weights of the adrenals in these pigeons injected with insulin and in similarly operated but uninjected pigeons were determined. Histological study of the hypothalamus, contents of the sella turcica and adrenals are being made.

Major findings: In the initial experiments, insulin caused a marked increase in adrenal weight when only the pars distalis was removed.

Significance to NIAMD research: The findings will contribute to the growing field of comparative endocrinology and to knowledge of the evolution of the endocrine system.

Proposed course of project: Initial observations will be augmented and extended to include tests following the removal of the

neurohypophysis alone and following lesions in the brain itself. The hypothalamus and unremoved parts of the hypophysis will be studied histologically and the findings will determine the course of experiments.

Project Description (Part 2):

Objective: To develop a sensitive method for the assay of prolactin in blood and urine.

Methods employed: Prolactin was injected intravenously into normal or hypophysectomized pigeons in a divided dose, two hours apart. Animals were killed four hours after the initial injection and the number of mitoses (arrested in metaphase by colchicine) counted in standard areas of whole amounts of the epithelium of the crop sacs.

Major findings: The initial results show that mitosis was increased by a total dose of 0.02 units (1.0 ug.) of prolactin, but not proportionately increased by doses 10 to 100 fold greater.

Significance to NIAMD research: It is obviously desirable to be able to detect and assay prolactin in blood and urine of clinical and experimental material. The local crop method in current use is unsuitable for blood and urine extracts, since toxic substances and trauma are known to cause a response simulating that of prolactin.

Proposed course of project: Modifications of the method will be explored to find a procedure where the response is in proportion to the dose of prolactin. Tests will be extended to include extracts of blood and urine.

Part B not included.

PHS - NIH
Individual Project Report
Calendar Year 1959

NIAMD - 16
1. Nutrition and Endocrinology
2. Fractionation and Isolation
3. Bethesda

Part A.

Project Title: Studies on Folic Acid

Principal Investigators: M. Silverman and J. C. Keresztesy

Other Investigators: R. Gardiner, K. O. Donaldson, R. Kisliuk

Cooperating Units: None

Man Years (Calendar Year 1959):
Total: 5
Professional: 4
Other: 1

Project Description:

Studies on folic acid:

(a) Naturally-occurring forms of folic acid (prefolic).

(b) Enzymatic conversion of prefolic acid to known forms.

(c) Delineation of the reaction sequence (in the rat) for the conversion of formimino-carbon (from formiminoglutamate) to the methyl level.

(d) Elucidation of the pathway of the synthesis of methionine in Escherichia coli.

Major findings: One of the naturally occurring forms of folic acid, prefolic A, has been isolated from horse liver. Its chemistry is being investigated. The enzymatic system which converts it to tetrahydrofolic acid was found to require catalytic amounts of FAD and a suitable hydrogen acceptor. Thus, it appears that prefolic A must be oxidized in order to be converted to tetrahydrofolic acid.

Rats fed small doses of ethionine for several weeks, excrete uroconic acid in the urine. This apparently is a result of inhibition of uroconase synthesis in liver. As a consequence of these observations, a search was made for the occurrence of uroconic acid in the urine of humans with liver disorders. This metabolite of histidine has been found in the urine of two (2) cirrhotics (in collaboration with Drs. Merritt and Rudnagel, NIMH).

(In collaboration with Dr. D. D. Brown, NIMH.) Methionine influences the metabolism of histidine by enabling the metabolism of formiminoglutamic acid to occur in folic acid and vitamin B_{12} deficient rats. There is an increased incorporation of lable from L-histidine-2-C^{14} into expired CO_2 from L-histidine-2-C^{14}. Homocystaine administration reduces the urinary formiminoglutamic acid, but does not alter the radioactive CO_2. No differences in the metabolism of histidine could be demonstrated in the two groups of rats (i. e., folic acid vs. vitamin B_{12} deficient).

It has been found that vitamin B_{12} does not activate folic acid in the rat (i. e., vitamin B_{12} deficiency was without influence on the conversion of folic acid to tetrahydrofolate derivatives in the intact animal).

A factor required for the synthesis of methionine (from homocysteine and serine) has now been purified 20 fold from extracts of E. coli and appears to be a vitamin B_{12} protein. This B_{12} protein has no effect on the activity of two other tetrahydrofolic acid dependant enzymes, namely, serine hydroxymethylase and hydroxymethyltetrahydrofolic acid dehydrogenase. It thus seems to be required specifically for methionine synthesis. The B_{12} protein is insensitive to light and cyanide treatment in contrast with the B_{12} coenzyme reported by Barker and co-workers. The B_{12} coenzyme does not replace the B_{12} protein in the methionine synthesizing system.

Significance to NIAMD research: The isolation and identification of the various metabolites of folic acid will result in a better understanding of the biochemistry of the anemias and leukemias. The mechanisms by which folic acid derivatives influence the formation of purines are being widely studied. This project is directed at discovering how the various forms of folic acid exist in the body and how they are changed from inactive to active forms.

Proposed course of project:

(1) To isolate the two forms of prefolic acid and establish their chemical structure.

Rats fed small doses of ethionine for several weeks, excrete urocanic acid in the urine. This apparently is a result of inhibition of urocanase synthesis in liver. As a consequence of these observations, a search was made for the occurrence of urocanic acid in the urine of humans with liver disorders. This metabolite of histidine has been found in the urine of two (2) cirrhotics (in collaboration with Drs. Merritt and Rudnagel, NNMR).

(In collaboration with Dr. D. D. Brown, NIMH.) Methionine influences the metabolism of histidine by enabling the metabolism of formiminoglutamic acid to occur in folic acid and vitamin B_{12} deficient rats. There is an increased incorporation of label from L-histidine-2-C^{14} into expired CO_2 from L-histidine-2-C^{14}. Homocystaine administration reduces the urinary formiminoglutamic acid, but does not alter the radioactive CO_2. No differences in the metabolism of histidine could be demonstrated in the two groups of rats (i. e., folic acid vs. vitamin B_{12} deficient).

It has been found that vitamin B_{12} does not activate folic acid in the rat (i. e., vitamin B_{12} deficiency was without influence on the conversion of folic acid to tetrahydrofolate derivatives in the intact animal).

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Significance to NIAMD research: The isolation and identification of the various metabolites of folic acid will result in a better understanding of the biochemistry of the anemias and leukemias. The mechanisms by which folic acid derivatives influence the formation of purines are being widely studied. This project is directed at discovering how the various forms of folic acid exist in the body and how they are changed from inactive to active forms.

Proposed course of project:

(1) To isolate the two forms of pteroylglutamic acid and establish their chemical structure.

(2) To study the reactions concerned in the transformation of prefolic acid to citrovorum factor and to purify the enzyme.

(3) To attempt to establish, at the biochemical level, the inter-relationship of folic acid and vitamin B_{12}.

(4) Determination of the mechanism by which methionine in the rat activates the utilization of the C-1 unit of formiminoglutamic acid.

Part B included.

Part B:

Publications other than abstracts from this project:

Donaldson, K. O., and Kerr-Grassy, J. C.

Naturally occurring forms of folic acid. I. "Prefolic A": Preparation of concentrate and enzymatic conversion to citrovorum factor. J. Biol. Chem. 234: 3235-3240, 1959.

PHS - NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD- 17
1. Nutrition & Endocrinology
2. Fractionation & Isolation
3. Bethesda

Part A.

Project Title: Microbiological assays for vitamins and amino acids.

Principal Investigator: Milton Silverman

Other Investigators: E. Bahrman and M. Ramirez

Cooperating Units: None

Man Years (Calendar Year 1959):

Total:	3.5
Professional:	1.5
Other	2

Project Description:

Objective: The determination of various vitamins and amino acids in foodstuffs, body tissues and excretory products can be carried out by the use of microbiological assays. Samples submitted by investigators on nutritional and allied projects are analyzed for their content of the specific vitamin or amino acid under study. New procedures are developed as required.

Methods employed: The use of microbiological assays continues to be of major importance in the studies on folic acid. For example, with suitable test conditions one can distinguish between folic acid and its reduced derivatives. Further, the concentrations of 5- and 10-formyl derivatives of tetrahydrofolic acid in milli-microgram amounts can be determined in the presence of each other and in the presence of tetrahydrofolic acid.

Major findings: Methods available for the determination of folic acid in animal tissues depend on (1) the enzymatic conversion of the tissue bound forms to microbiologically available forms and (2) measurement of the latter by microbiological methods. Current findings indicate that by the

use of suitable extraction procedures and the proper assay organism (L. casei) the enzymatic procedure may be eliminated. These findings should prove of value in assaying tissues which have a low content of folic acid derivatives.

Significance to NIAMD research: These microbiological assay procedures are a very important tool for the measurement of the concentrations of various cell constituents, i. e., amino acids and vitamins.

Proposed course of project: To continue current microbiological problems and collaborate when called upon with other groups on projects requiring microbiological assays of vitamins and amino acids.

Part B included.

Part B:

Publications other than abstracts from this project:

Windmueller, H. G., Ackerman, C. J., Bakerman, E., and Mickelsen, O. Reaction of ethylene oxide with nicotinamide and nicotinic acid. J. Biol. Chem. 234, 889-893, 1959.

PHS - NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD- 18
1. Nutrition & Endocrinology
2. Fractionation & Isolation
3. Bethesda

Part A.

Project Title: Large-scale processing of biological material.

Principal Investigator: John C. Keresztesy

Other Investigators: None

Cooperating Units: NIH - Natural Products Laboratory

Man Years (Calendar Year 1959):
Total: 1.5
Professional: 0.50
Other: 1.00

Project Description:

Objectives: Many problems of importance in the biochemistry of disease require the isolation and identification of substances which are present in only trace amounts in the natural product. It becomes necessary, in order to obtain sufficient quantities of the desired compound, to process large amounts of biological materials, such as liver, brains, excretory products, plant materials, etc.

Methods employed: The laboratory is equipped with large-scale apparatus, such as stills, filters, reaction and extraction kettles, etc. In most isolation problems the original small-scale process has to be modified and developed, so that it can be carried out efficiently on the larger scale. This adaptation or process development is an important function of the laboratory. The degree of participation of the laboratory varies according to the needs of the specific problems.

Major findings: The large-scale laboratory facilities continue to be used for an increasing number of NIH investigations.

Several tons of horse liver were processed for prefolic A concentrates. A variety of plant materials were extracted for the Natural Products Laboratory (NIH). There were greater demands for large batch (300 L.) fermentations. A modification of the piping of the equipment removed a source of contamination.

Significance to NIAMD research: Ordinary laboratories lack facilities to effectively carry on studies which require the extraction and processing of large quantities of biological materials. However, with the equipment and trained personnel of the large-scale laboratory, such investigations as the isolation of the prefolic acids can be undertaken and successfully completed.

Proposed course of project: To continue to offer assistance in large-scale operations as required by such projects as prefolic acid and the alkaloid problem (NIH).

Part B not included.

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD-10
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: Carbohydrate Metabolism

Principal Investigator: Dr. Gilbert Ashwell

Other Investigators: Dr. Jean Hickman
Mr. William Fricer

Cooperating Units: Dr. J. D. Smiley, Research Associate
Dr. M. A. Cynkin, Cancer Fellow
Dr. J. J. Burns, National Heart Institute, Laboratory of Chemical Pharmacology

Man Years (calendar year 1959):
Total: 5 years
Professional: 4 1/3 years
Other: 2/3 year

Project Description:

Objectives: The present studies are an outgrowth of earlier work on the metabolism of uronic acids in mammalian and bacterial tissues as well as an investigation of the biosynthesis and degradation of ascorbic acid. This work has been extended to cover the mechanism of formation of some of the more complex sugars found in mammalian mucopolysaccharides and in the endotoxin forming lipopolysaccharides of bacterial origin.

Methods Employed: In general, the methods employed involve the use of radioactive incorporation of precursor sugars into the compounds being studied. This is usually followed by participation of the enzyme system involved. Intermediates are determined by specific enzymatic or colorimetric procedures and isolated by column or paper chromatography. Final identification is made by the preparation of appropriate crystalline derivatives.

Part B included X Yes ____ No

Major Findings: The outlines of a new pathway for uronic acid metabolism in bacteria were recorded in the last annual report. This pathway has been intensively studied during the ensuing year and purification of all the enzymatic reactions achieved. This work has been completed and has been submitted for publication.

Studies on the biosynthesis of L-iduronic acid in animal tissues have been undertaken in conjunction with Dr. Jean Hickman. Preliminary results have shown that mouse skin homogenates contain an enzymatic system capable of incorporating variously labelled sugar precursors into chondroitin sulfate B, an iduronic acid containing mucopolysaccharide. Comparison of the relative efficiency of incorporation revealed glucuronic-G-C^{14} to be a far better precursor than UDPGA-6-C^{14} and UDPG-6-C^{14} to be completely inert. These unexpected findings cannot be readily explained on the basis of our present knowledge and suggest the presence of a heretofore unrecognized pathway of glucuronic acid metabolism. This presumption has been somewhat strengthened by the very recent observation that the same enzyme preparation catalyzes a rapid exchange of P^{32}-P^{32} in the presence of glucuronic acid-1-phosphate and a mixture of nucleotide diphosphates.

An alternate approach to the problem of L-iduronic acid metabolism has been undertaken together with Dr. James Smiley and, more recently, with Mr. William Pricer. These studies have shown that chemically synthesized L-iduronic acid is rapidly metabolized in the presence of TPNH by both liver and kidney preparations with the resultant formation of L-idonic acid. This enzyme, which has been purified about 200-fold from beef kidney is not specific for L-iduronic acid. However, the determinations indicate the affinity of the enzyme for this substrate to be significantly greater than that for any other compound so far examined. The subsequent metabolism of the L-idonic acid formed is under investigation.

In a continuing collaboration with Dr. Burns and Mr. Kanfer of the Heart Institute, further progress on the enzymatic degradation of ascorbic acid in mammalian tissues has been made. A partially purified rat kidney enzyme has been obtained which catalyzes the decarboxylation of diketogulonic acid, a naturally occurring oxidation product of ascorbic acid. The products of this reaction have been identified as L-lyxonic and L-xylonic acid. L-xylonic-1-C^{14} and L-lyxonic-1-C^{14} have been synthesized and examined for further metabolism by *in vivo* experiments with rats. Less than 5% of injected C^{14} appeared in the expired CO_2

Major Findings, cont.

in the first 24 hours, the bulk of the radioactivity being recovered in the urine. At the present time, neither the mechanism nor the biological significance of this reaction is known.

An alternate enzymatic degradation of diketogulonic acid in mammalian kidney has been observed by Dr. Smiley. In this case, a DPN-linked dehydrogenase catalyzes the formation of 2-keto-L-gulonic acid. This enzyme has been purified free from contamination with the above described decarboxylase. The significance of this finding, as well as the subsequent fate of the 2-keto hexonic acid, is being pursued.

In collaboration with Dr. Morris Cynkin, an investigation of the biosynthesis of a new group of rare sugars, the 3,6 dideoxy hexoses, has been initiated. These sugars have been shown to be present in the endotoxins (o-antigens) of Salmonella and Escherichia and appear to be located on the ends of polysaccharide chains. They have been demonstrated to be involved in the immunological specificity of the o-antigens.

A modification of the malonaldehyde-thiobarbituric acid reaction has been developed which permits the detection and quantitative determination of these sugars in quantities of less than 0.01 μmoles. Preliminary results of growth experiments involving glucose-1-C^{14} indicate that glucose is converted to 3,6-dideoxy-L-xylohexose (colitose) in E. coli 0111 without rearrangement or inversion of the carbon skeleton.

Significance to NIAMD Research: Knowledge of the metabolic processes of the sugars and sugar acids described in this report is essential for the understanding of the mechanism of formation of the complex polysaccharides and their role in normal and pathological states. The present studies are directed toward an understanding of the basic problems of mucopolysaccharide biosynthesis. Only in the light of this knowledge can a rational approach to the more specific problems of pathology be made.

Proposed Course of Project: It is planned to continue along the outlines described above in the expectation that relevant information concerning the biosynthesis and metabolism of the biologically important muco- and lipopolysaccharides will be obtained.

Part D.

Publications during 1959:

Burns, J. J. and Ashwell, G.: L-Ascorbic acid, the enzymes, edited by Lardy, et al. Academic Press, in press.

Ashwell, G., Kanfer, J., and Burns, J. J.: Studies on the mechanism of L-xylulose formation by kidney enzymes. J. Biol. Chem. 236: 472, 1959.

Kanfer, J., Burns, J. J., and Ashwell, G.: L-Ascorbic acid synthesis in a soluble enzyme system from rat liver microsomes. Biochim. Biophys. Acta 31: 556, 1959.

Hickman, J. and Ashwell, G.: A sensitive and stereospecific enzymatic assay for xylulose. J. Biol. Chem. 234: 758, 1959.

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD-20
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: Chemical and Enzymic Studies Related to the Structure and Metabolism of Ribonucleic Acid and its Constituents.

Principal Investigator: Dr. Maxine F. Singer

Other Investigators: Dr. Audrey Stevens

Cooperating Units: Dr. Giulio L. Cantoni, Laboratory of Cellular Pharmacology, National Institute of Mental Health (Serial No. M-CP22)

Man Years (calendar year 1959):
Total: 1 1/3 years
Professional: 1 year
Other: 1/3 year

Objectives: The object of the main part of the work in the year 1958 was to study enzymes that catalyze the breakdown of ribonucleic acid and then to use some of these enzymes, in conjunction with chemical methods, to elucidate the structure of the so-called "soluble" ribonucleic acid that is presumably involved in protein biosynthesis. Recently studies have been begun on the biosynthesis of 5-ribosyluracil-5'-monophosphate, a newly discovered constituent of RNA.

Methods Employed: Column and paper chromatographic techniques have been utilized to study the products of RNA degradation. Assays of enzymic activities have involved chemical and isotope tracer techniques. Tracer methods are also being used to study 5-ribosyluracil biosynthesis.

Major Findings: A new nuclease has been discovered in extracts of mouse, Ehrlich Ascites Tumor cells. This nuclease has been partly purified and shown to hydrolyze polyadenylic and polyuridylic acids to 5'-AMP and 5'-UMP, respectively.

Part B included X Yes ___ No

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD-20
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: Chemical and Enzymic Studies Related to the Structure and Metabolism of Ribonucleic Acid and its Constituents.

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Other Investigators: Dr. Audrey Stevens

Cooperating Units: Dr. Giulio L. Cantoni, Laboratory of Cellular Pharmacology, National Institute of Mental Health (Serial No. M-CP22)

Man Years (calendar year 1959):
Total: 1 1/3 years
Professional: 1 year
Other: 1/3 year

Objectives: The object of the main part of the work in the year 1958 was to study enzymes that catalyze the breakdown of ribonucleic acid and then to use some of these enzymes, in conjunction with chemical methods, to elucidate the structure of the so-called "soluble" ribonucleic acid that is presumably involved in protein biosynthesis. Recently studies have been begun on the biosynthesis of 5-ribosyluracil-5'-monophosphate, a newly discovered constituent of RNA.

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Major Findings: A new nuclease has been discovered in extracts of mouse, Ehrlich Ascites Tumor cells. This nuclease has been partly purified and shown to hydrolyze polyadenylic and polyuridylic acids to 5'-AMP and 5'-UMP, respectively.

Part B included X Yes ___ No

Major Findings, cont.

The alkaline degradation of "soluble" RNA from rabbit liver has yielded adenosine and 3',5'-guanosine diphosphate (mixed with the 2',5'-isomer) (G_{p_p}) as well as the 2' and 3' isomers of the four usual mononucleotides. This indicates that the chains in this preparation are terminated, at the "nucleoside" end by adenosine, and by 5'-guanylic acid at the other end. The approximate ratios of the total amounts of adenosine, uridine, guanosine, cytidine and 5-ribosyluracil in this soluble RNA were found to be 1:1:1.8:1.7:0.2.

Extensive work on the mechanism of polynucleotide phosphorylase action on polyribonucleotides in previous years allowed the use of this enzyme to elucidate aspects of "soluble" RNA structure. Soluble RNA is phosphorolyzed very slowly by polynucleotide phosphorylase. Moreover, the reaction stops when from 20 to 30 percent of the "soluble" RNA has been converted to nucleoside diphosphates. Our previous work had demonstrated that polynucleotide phosphorylase acts on a polynucleotide chain by stepwise removal of mononucleotide units starting at the "nucleoside" end of the chain (that end bearing unesterified C-2' and C-3' hydroxyls). Since it is also known that amino acids are bound to "soluble" RNA through either this C-2' or C-3' hydroxyl group, it was expected that the ability of "soluble" RNA to act as an acceptor for amino acids would be altered by polynucleotide phosphorylase. It was found, however, that polynucleotide phosphorylase does not destroy the amino acid acceptor ability of "soluble" RNA after 20-30 percent phosphorolysis. The amino acid acceptor activity per nucleotide residue of "soluble" RNA is unchanged for several amino acids. Preliminary experiments suggest that the nature of the secondary structure of the chains may determine their resistance to degradation.

It has been found that growing yeast cells utilize orotic acid as a precursor for 5-ribosyluracil.

Significance to NIAMD Research: These studies contribute to our knowledge of the structure and synthesis of the "soluble" RNA and other RNAs. Thereby, they are directly concerned with the mechanism of protein synthesis, as well as RNA structure and consequently are significant for many problems concerning normal or abnormal cellular function.

Proposed Course of Project: The purification and study of the ascites nuclease will be continued as a means toward a new tool for the study of RNA structure. The studies on polynucleotide phosphorylase action on "soluble" RNA will be continued in order to determine precisely the structural features responsible for its resistance to enzymic attack. In addition, it is planned to use other enzymes to elucidate further the structural features of "soluble" RNA.

The studies on 5-ribosyluracil biosynthesis are being continued with *in vitro* experiments.

Part B.

Publications during 1959:

Singer, M. F., Heppel, L. A., Hilmoe, R. J., Ochoa, S., and Mii, S.: Enzymatic synthesis of polyribonucleotides. In Begg, R. W. (ed.): Proceedings of the Third Canadian Cancer Conference. New York, Academic Press, Inc., 1959, p. 41.

Heppel, L. A., Singer, M. F., and Hilmoe, R. J.: The mechanism of action of polynucleotide phosphorylase. Ann. N. Y. Acad. Sci. 81: 635, 1959.

Singer, M. F., Heppel, L. A., and Hilmoe, R. J.: Oligonucleotides as primers for polynucleotide phosphorylase. J. Biol. Chem., in press.

Singer, M. F., Hilmoe, R. J., and Heppel, L. A.: The polymerization of guanosine diphosphate by polynucleotide phosphorylase. J. Biol. Chem., in press.

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD-21
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: Studies on the Structure, Biosynthesis and Intermediary Metabolism of Nucleic Acids and Small Nucleotides.

Principal Investigator: Dr. Leon A. Heppel

Other Investigators: Dr. Russell J. Hilmoe
Dr. Maria Lipsett

Cooperating Units: Dr. Audrey Stevens
Dr. E. P. Anderson (National Cancer Institute) NCI-427b

Man Years (calendar year 1959):
Total: 3 years 7 months
Professional: 2 3/4 years
Other: 10 months

Project Description:

Objectives: The object of this project is to discover pathways for the biosynthesis and breakdown of nucleic acids and smaller polynucleotides and to discover features of the structure of RNA that are important for its function.

Methods Employed: Biosynthesis and degradation of RNA and other polynucleotides is studied, using enzymes purified from bacterial, plant and animal sources. The reactions are followed by means of paper and column chromatography, chemical analysis and isotope tracer methods. Also, purified enzymes are used as specific analytical reagents for study of polynucleotide structure. Physical methods for study of macromolecular structure include spectrophotometric measurement, ultra centrifugation, optical rotation and infrared spectroscopy.

Major Findings: In the field of nucleic acids, ever-increasing importance is being attached to the matter of secondary structure, which means the secondary aggregation of

Part B included X Yes ___ No

Major Findings, cont.

individual polynucleotide chains. The forces holding these chains together are hydrogen bonds between certain pairs of bases. This is how the double helix of DNA and the double helix consisting of poly A + poly U are formed. Specific hydrogen bonding also explains enzymic replication of DNA, and probably of RNA. Several significant observations in this field were made during the current year.

The most striking result, by Dr. Lipsett, was the formation of a regular, triple-stranded structure between the polymer, polyadenylic acid and a uridine containing tetranucleotide. At a slower rate, interaction was observed even with a trinucleotide. The reverse interaction, between polyuridylic acid and the adenosine containing tetranucleotide, pApApApA, has also been observed. These interactions proceed to the same extent and have all of the features of hydrogen bonding between two large polymers except that they fall apart or "melt out" at less elevated temperatures. The question of how large a molecule must be before a double helix can be formed is considered to be a crucial one.

In other work (L. A. Heppel) it was found that the enzyme polynucleotide phosphorylase is subject to powerful and highly specific inhibitory effects. Any nucleoside diphosphate can be polymerized in the presence of any polymer with the following exceptions: (1) Polyadenylic acid specifically inhibits UDP and IDP polymerization. (2) Polyuridylic acid inhibits only ADP polymerization. (3) Polycytidylic acid inhibits only IDP polymerization. (4) Polyinosinic acid inhibits CDP and ADP. The exchange of radioactive phosphate with nucleoside diphosphate is also subject to the same specific inhibitory effects. Only certain combinations are involved, and they represent those pairs of bases which can hydrogen-bond. These experiments provide strong evidence that the action of this enzyme is not a purely random process, but subject to real restrictions which could govern synthesis of a specific RNA.

A new nuclease was discovered in Azotobacter agilis extracts, purified and its mechanism of action investigated (Stevens and Hilmoe). The enzyme is proving very useful in studies of S-RNA structure. A phosphodiesterase was purified from leukemia cell extracts and found to exhibit a new form of specificity, for it hydrolyses nothing smaller than a dinucleotide. Other phosphodiesterases hydrolyse simple esters of nucleotides as well.

Significance to NIAMD Research: The present studies help to clarify mechanisms for RNA synthesis and utilization. They also give new information on the chemical and the macromolecular structure of polynucleotides. Consequently they are of significance for problems of hereditary mechanisms, certain metabolic diseases, and plant and animal viruses. Governing principles coming out of this work also apply, to some extent, for DNA synthesis. Finally, the work pertinent to S-RNA is important in protein biosynthesis.

Proposed Course of Project: Further studies on secondary structure of interacting polymers and of RNAs are contemplated. The specificities of polynucleotide phosphorylase will continue to be explored. Studies on the structure of S-RNA will be pursued, making particular use of specific nucleases and phosphodiesterases as analytical reagents.

Part B.

Honors: Appointment to the Board of Editors, Journal of Biological Chemistry.

Publications during 1959:

Strominger, J. L., Heppel, L. A., and Maxwell, E. S.: Nucleoside monophosphate kinases I. Transphosphorylation between adenosine triphosphate and nucleoside monophosphates. Biochim. et Biophys. Acta 32: 412, 1959.

Heppel, L. A., Strominger, J. L., and Maxwell, E. S.: Nucleoside monophosphate kinases II. Transphosphorylation between adenosine monophosphate and nucleoside triphosphates. Biochim. et Biophys. Acta 32: 422, 1959.

Shuster, L., Khorana, H. G., and Heppel, L. A.: The mode of action of ryegrass ribonuclease. Biochim. et Biophys. Acta 33: 452, 1959.

Heppel, L. A., Singer, M. F., and Hilmoe, R. J.: Mechanism of action of polynucleotide phosphorylase. Proc. of the New York Academy of Sciences 81: 635, 1959.

Hilmoe, R. J.: The effect of endgroups and the initial site of attack on polynucleotides by polynucleotide phosphorylase and certain phosphodiesterases. Proc. of the New York Academy of Sciences 81: 660, 1959.

Singer, M. F., Hilmoe, R. J., and Heppel, L. A.: The polymerization of guanosine diphosphate by polynucleotide phosphorylase. J. Biol. Chem., to appear March 1960.

Singer, M. F., Heppel, L. A., and Hilmoe, R. J.: Oligonucleotides as primers for polynucleotide phosphorylase. J. Biol. Chem., to appear March 1960.

Heppel, L. A.: 5'-Nucleotidase. In Boyer, P. D., Lardy, H., and Myrbäck, K. (eds.): The Enzymes. New York, Academic Press, Inc., 1960, Vol. III, in press.

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD- 22
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: Gene-Enzyme Relationships in Histidine Biosynthesis.

Principal Investigator: Dr. Bruce N. Ames

Other Investigators: Barbara Garry

Man Years (calendar year 1959):
Total: 1 1/2 years
Professional: 1 year
Other: 1/2 year

Objectives:

1. The genes controlling histidine biosynthesis. Hartman of Johns Hopkins has mapped the genes of histidine biosynthesis in Salmonella and has found they are all in a cluster on the Salmonella chromosome. We have set up enzyme assays for the different enzymes of the pathway so as to determine which mutants are missing which enzymes and to see if there is any correlation between the sequences of the genes on the chromosome and the sequence of the enzymes they control in the biosynthetic pathway.

2. Repression of the histidine biosynthetic enzymes by histidine. We have investigated the repression by histidine of the synthesis of the enzymes of histidine biosynthesis. Two points were under investigation: a) Does the histidine repression of enzyme synthesis affect each of the enzymes of the pathway to the same extent. b) Is there any influence of the concentration of the enzyme substrates on the enzyme synthesis control mechanism.

Methods Employed: We have used various histidine mutants of Salmonella isolated by Dr. P. E. Hartman and modified the assays for the enzymes of histidine biosynthesis we have previously described in Neurospora.

Part B included ___ Yes X No

Major Findings:

1. The biochemical analysis of Hartman's mutants has indicated that, in general, each genetic class of mutants can be associated with the loss of a particular biosynthetic enzyme. The sequence of the histidine genes on the chromosome linkage map corresponds to the sequence of the enzymes they control in the biosynthetic pathway. In addition, certain of the mutants which behave genetically as if they were missing a section of the chromosome covering all of the histidine genes have been shown to be missing all four of the biosynthetic enzymes that were tested for.

2. It has been possible, by lowering the histidine pool in Salmonella to raise the level of the histidine biosynthetic enzymes about 35-fold over the level of these enzymes in Salmonella growing on minimal medium. One way of doing this is to grow a histidine-requiring mutant on a derivative of histidine as a source of histidine so that the growth rate is limited by the amount of histidine available to the organism. By using mutants blocked at different points in the pathway we have been able to show that histidine alone controls the rate of synthesis of the various enzymes of the pathway, that the histidine repression is independent of the quantity of each enzyme substrate present in the cells. Depending on the mutant, an enzyme can be in the presence of substrate or no substrate without influencing the repression of its synthesis by histidine.

The major finding of this study of repression was that histidine affects the synthesis of each of the enzymes of the pathway to the same extent. This phenomenon has been called coordinate repression. Several other enzymes (glutamic dehydrogenase and histidine activating enzyme) have been shown not to be influenced by the size of the histidine pool in the organism. Many hypotheses would be consistent with the finding that histidine represses the synthesis of all of the histidine biosynthetic enzymes together. One attractive possibility which is suggested is that, as the histidine genes are closely linked on the chromosome, this feedback mechanism may work at the gene level and histidine (or a histidine-nucleic acid repressor) has a specific affinity for the histidine section of the chromosome and can "turn off" these genes when the internal histidine concentration rises.

Significance to NIAMD Research: The control mechanisms of the cell are of fundamental importance. The problem of the genetic control of metabolism is one of the central ones of biology and has implications for all of medical science. An understanding of how genes are "turned on and off" may be the key to the problem of differentiation.

Proposed Course of Project: In Neurospora and yeast it has been shown that the histidine genes are scattered on the chromosome and it will be of interest to see if repression is possible in these organisms. Further work is planned to try and characterize the repressor in Salmonella and to study its mode of action.

PHS-NIH
Individual Project Report
Calender Year 1959

Serial No. NIAMD-23
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: The Role of Polyamines in the Neutralization of Bacteriophage DNA.

Principal Investigator: Dr. Bruce N. Ames

Other Investigators: Dr. Donald T. Dubin

Man Years (calendar year 1959):
Total: 1 year
Professional: 1 year
Other: None

Project Description:

Objectives: It was previously reported that bacteriophage T4 contained the polyamines putrescine, $NH_2(CH_2)_4NH_2$, and spermidine, $NH_2(CH_2)_3NH(CH_2)_4NH_2$, in amounts sufficient to neutralize about half of the viral DNA. The putrescine and spermidine in the phage were found to be derived from the large amount of these polyamines normally present in the host bacterium, Escherichia coli. It was also shown that these cations were the unidentified compounds in phage T2 reported by Hershey to be injected into the bacteria along with the viral DNA.

These findings on polyamines in phage raised several questions:

1) Is the role of the polyamines in phage that of specific or non-specific cations for neutralizing the negatively charged phosphate groups in the DNA?

2) Are the amounts and kinds of polyamines in the phage determined by the phage or by the bacterial pool of cations?

3) Can stoichiometry between cations in the phage and the phosphate anions of the DNA be demonstrated?

4) What is the distribution of polyamines in viruses?

Part B included X Yes ___ No

Methods Employed: Various viruses were grown and purified and then assayed for polyamines and phosphate.

Major Findings: The cations of T4 phage have been examined and a balance has been obtained between total cations and total DNA anions. The cations putrescine^{++}, spermidine^{+++}, and Mg^{++} neutralise the DNA of the T4 bacteriophage obtained from E. coli grown in minimal medium. The cations in T4 phage have been shown to be a function of both the composition of the pool of cations in the host bacterium at the time of phage assembly and the affinity of each species of cation for the phage nucleic acid. Viable T4 phage have been obtained with various cations as the DNA-neutralizing agent; the role of the polyamines in phage appears to be that of a non-specific cation for DNA neutralization and stabilization.

The absence of polyamines in certain E. coli and S. typhimurium phages was correlated with their permeability to cations; it appears as if the polyamines are displaced by other cations during purification of the phage. Polyamines are not present in TMV, Cucumber Virus, Tomato Bushy Stunt Virus, or Polio Virus.

Significance to NIAMD Research: The present studies help to clarify the role of the polyamines as DNA neutralizing agents. Consequently they are of significance for problems of hereditary mechanisms and the structure of viruses.

Proposed Course of Project: The objectives of the study have been realized and no further work is planned at this time.

Part B.

Publications during 1959:

Ames, B. N. and Dubin, D. T.: The role of polyamines in the neutralization of bacteriophage DNA. J. Biol. Chem., in press.

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD-24
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: Enzymatic Utilization of Model Compounds

Principal Investigator: William B. Jakoby

Other Investigators: Gerald D. Aurbach

Cooperating Units: Dr. E. W. Yamada, Fellow of the Jane Coffin Childs Memorial Fund for Medical Research
Dr. M. Nirenberg, Fellow of the American Cancer Society
Dr. Wayne Albers, NINDB: MA-NC-8

Man Years (calendar year 1959):
Total: 3
Professional: 2 1/2
Other: 1/2

Project Description:

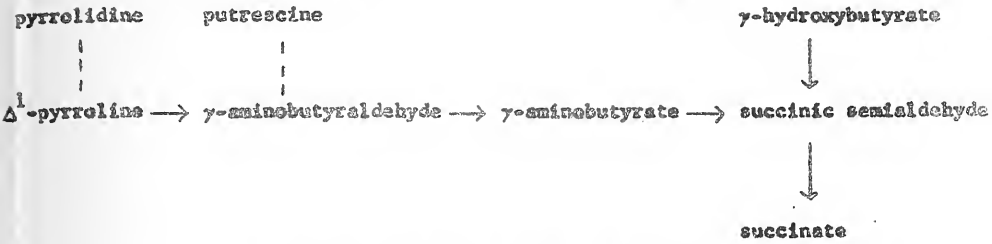
These investigations were concerned with the reactivity of various chemical groupings in enzyme catalyzed reactions. The compounds studied most intensively have been γ-aminobutyric acid and acetylenemonocarboxylic acid.

Methods Employed: By use of the enrichment culture technique, microorganisms were obtained with the ability to grow on various model compounds as sole carbon source. Enzymes from these organisms were investigated by the usual techniques.

Major Findings: The biosynthesis of γ-aminobutyrate from pyrrolidine and putrescine and the subsequent utilization of this compound have been studied at the enzyme level. Each of the enzyme reactions which are denoted by solid lines have been purified and characterized and evidence for each of the intermediates listed has now been obtained.

Part B included X Yes ___ No

Major Findings, cont.



Of particular interest has been the study of the kinetics of one of these reactions, the transamination of γ-aminobutyrate and γ-ketoglutarate resulting in the formation of succinic semialdehyde and glutamate. The data had suggested that transamination occurred by way of a series of binary complexes of enzyme and each substrate. Further support for this concept has been obtained by the dissection of the transamination into two exchange reactions.

Continuing studies on the mechanism of enzyme catalyzed aldehyde oxidation have resulted in information concerning the sites of substrate binding to the protein. Employing techniques of enzyme digestion and competitive inhibition it has been concluded that aldehyde substrates are bound to closely juxtaposed SH groups of the enzyme, whereas pyridine nucleotides are bound at sites other than sulfhydryl groups. In a study of a novel aldehyde dehydrogenase oxidizing malonic semialdehyde, both DPN and CoA were found to be involved, resulting in the direct formation of carbon dioxide and acetyl-CoA.

Significance to NIAMD Research: Each of the compounds studied impinges on an area of vital interest in the fields of biology and medicine. A particularly clear example of basic research leading to practical application may be cited from the above-noted work on γ-aminobutyric acid metabolism which has led to an extremely sensitive and specific method for the determination of this compound in brain where it appears to play a role in both nervous and metabolic activity.

Proposed Course of Project: It is expected that the mechanism of aldehyde oxidation by enzymes will be further investigated. A study of the metabolism of other model compounds, e.g., glutaric acid and erythritol, will be continued.

Part B.

Publications during 1959:

Scott, E. M. and Jakoby, W. B.: Soluble γ-aminobutyric-glutamic transaminase from Pseudomonas fluorescens. J. Biol. Chem. 234: 932, 1959.

Jakoby, W. B. and Scott, E. M.: Aldehyde oxidation VII. Succinic semialdehyde dehydrogenase. J. Biol. Chem. 234: 937, 1959.

Jakoby, W. B. and Fredericks, J.: Pyrrolidine and putrescine metabolism: γ-Aminobutyraldehyde dehydrogenase. J. Biol. Chem. 234: 2141, 1959.

Yamada, E. W. and Jakoby, W. B.: Enzymatic utilization of acetylenic compounds II. Acetylenemonocarboxylic acid hydrase. J. Biol. Chem. 234: 941, 1959.

Jakoby, W. B. and Marred, S. A.: Aldehyde oxidation IV. An aldehyde buffer for growth studies. J. Bact. 77: 410, 1959.

Jakoby, W. B. and Yamada, E. W.: Direct enzymic conversion of malonic semialdehyde to acetyl-coenzyme A. Biochim. Biophys. Acta 34: 276, 1959.

Hirenberg, M. and Jakoby, W. B.: Enzymatic utilization of γ-hydroxybutyric acid. J. Biol. Chem., in press.

Jakoby, W. B.: Enzymatic formation and utilization of γ-aminobutyric acid in Pseudomonas. In Roberts, E. (ed.): Inhibition in the Nervous System and γ-Aminobutyric Acid, in press.

Albers, W. R. and Jakoby, W. B.: Transamination and the isotopic labelling of glutamate in brain. In Roberts, E. (ed.): Inhibition in the Nervous System and γ-Aminobutyric Acid, in press.

Jakoby, W. B.: Enzymes of γ-aminobutyrate metabolism, bacterial. In Colowick, S. and Kaplan, N. O. (eds.): Methods in Enzymology, New York, Academic Press, Vol. IV, in press.

Publications during 1959, cont.

Yamada, E. W. and Jakoby, W. B.: Aldehyde oxidation V. Direct conversion of malonic semialdehyde to acetylcoenzyme A. J. Biol. Chem., in press.

Hayaishi, O., Slaughter, C., and Jakoby, W. B.: 3-Hydroxy bile acid dehydrogenase from Escherichia freundii. J. Bact., in press.

Jakoby, W. B.: Oxalate decarboxylation. In Colowick, S. and Kaplan, N. O. (eds.): Methods in Enzymology, New York, Academic Press, Vol. IV, in press.

Albers, W. R. and Jakoby, W. B.: Exchange reactions catalyzed by γ-aminobutyric-glutamic dehydrogenase. Biochim. Biophys. Acta, in press.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The Biosynthesis of Inositol

Principal Investigator: Dr. Frank Eisenberg, Jr.

Other Investigator: Dr. Yoh Imai

Cooperating Units: None

Man Years (calendar year 1959):
Total: 2-1/2
Professional: 2
Other: 1/2

Project Description:

Objectives - To elucidate the mode of biosynthesis of inositol in the mammal.

Methods Employed - Various carbon-labeled sugars and sugar derivatives were administered intraperitoneally to rats. Three hours later the animals were killed and myo-inositol was isolated from the whole animals and assayed for the amount of isotope. In some experiments the inositol was partially degraded by three different methods to allocate the isotope to one or at most two carbon atoms as follows:

(1) Oxidation by A. suboxydans to myo-inosose-2 followed by periodate oxidation to yield CO_2 (C-2).
(2) Oxidation by HNO_2 to D,L-epi-inosose-2 followed by periodate oxidation to yield CO_2 (C-4,6).
(3) Oxidation by a rat kidney supernatant to D-glucuronic acid followed by acid decarboxylation to yield CO_2 (C-1).

Major Findings - Of the various labeled six-carbon and smaller compounds administered to the rats glucose and galactose were found to be the best precursors of inositol. The extent of synthesis of isotopic inositol from both of these sugars was independent of the location of the label, suggesting that a six-carbon unit is the immediate precursor of inositol in the rat. This unit, however, is not glucuronic acid, since although glucuronic acid-U-C^{14} was incorporated, glucuronic acid-6-C^{13} was not.

These results indicate that the mode of biosynthesis of inositol in the rat is different from that observed in yeast where a two-carbon and four-carbon unit combine to form inositol. Furthermore, the cleavage of inositol to glucuronic acid observed in rat kidney extracts is not sufficiently reversible to account for inositol biosynthesis in the whole animal.

Partial degradation studies have supported the six-carbon cyclization mechanism. Glucose-1-C^{14} gives rise predominantly to C-4,6 labeled inositol; glucose-2-C^{14} to C-2,5; and glucose-6-C^{14} to C-1.

Significance to NIAMD Research - The presence of inositol in animal tissues both in the free state and in combination with lipide materials makes the study of its biosynthesis of interest and importance to a complete understanding of carbohydrate metabolism and its possible link to fat metabolism. Without an understanding of the normal course of these reactions a derangement in one or more of them might not be recognized.

Proposed Course of Project - The definitive conclusion that inositol is derived from a six-carbon unit must await the complete degradation of the compound. Chemical methods to achieve this end are now being tested and will then be applied to the biosynthetic labeled inositol.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Eisenberg, F., Jr., Dayton, P. G. and Burns, J. J. Studies on the glucuronic acid pathway of glucose metabolism. J. Biol. Chem. 234, 250-253 (1959).

Eisenberg, F., Jr. and Leder, I. G. An improved scanner for radioactive paper strips. Anal. Chem. 31, 627-628 (1959).

Dayton, P. G., Eisenberg, F., Jr. and Burns, J. J. Metabolism of C^{14}-labeled ascorbic, dehydroascorbic and diketogulonic acids in guinea pigs. Arch. Biochem. Biophys. 81, 111-118 (1959).

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Studies on the Degradation of Insulin and Insular Derivatives by Mammalian Tissues

Principal Investigators: Dr. Frank Tietze and Dr. Glenn E. Mortimer

Other Investigator: Dr. DeWitt Stetten, Jr.

Cooperating Units: Clinical Endocrinology Branch- NIAMD 150C

Man Years (calendar year 1959):
Total: 2-1/2
Professional: 2
Other: 1/2

Project Description:

Objectives - Previous studies in this project on the fate of insulin-I^{131} in intact, perfused rat liver have shown that this organ can carry out the extensive degradation of the labeled protein. Such degradation is presumably catalyzed by an enzyme system, termed insulinase, which has been found by other workers in soluble extracts of liver and other mammalian organs. More recent work with intact liver has been concerned with the specificity of the enzyme(s) comprising this degradative system and with the role of the cell membrane in the capture and degradation of insulin-I^{131} within the intact cell.

Methods Employed - Intact rat liver is cyclically perfused, at 37° or 0°, with oxygenated whole rat blood containing trace amounts of insulin-I^{131}. At intervals of time, aliquots of the perfusing medium are sampled for total radioactivity, TCA-soluble radioactivity (a measure of degraded insulin), and TCA-insoluble radioactivity (a measure of undegraded insulin). Such perfusions have been carried out with native and denatured insulin-I^{131} either alone or in the presence of possible competing substrates. Paper chromatographic methods have been employed in a number of experiments to determine the monoiodotyrosine (MIT) and diiodotyrosine (DIT) contents of undegraded insulin prior to and following perfusions.

Major Findings - Determination of the MIT and DIT contents of various preparations of insulin-I^{131} has indicated that the fraction of iodoinsulin resistant to perfused rat liver increases with increasing initial content of DIT in the labeled substrate. Furthermore, the DIT: MIT ratio of the resistant fraction of labeled protein is significantly higher than that of the substrate prior to perfusion.

The results of a number of experiments have suggested that the degradation of insulin-I^{131} by intact liver may proceed by a sequence of steps which, in the simplest case, may consist of the following events: 1) Binding of insulin by the cell membrane; 2) transport of insulin to the site of insulinase activity; 3) degradation of insulin. Thus, for example, when liver is perfused with insulin-I^{131} at 0^{o} a substantial uptake of the label is observed without, however, a concomitant appearance of TCA-soluble products; in contrast a soluble enzyme preparation obtained from rat liver possessed considerable insulinase activity when measured at 0^{o}.

Evidence for the role of the cell membrane as a determinant of the specificity of insulin degradation by intact rat liver has been furnished by a number of observations. Thus, whereas soluble insulinase preparations are capable of extensive degradation of alkali-denatured insulin-I^{131}, intact liver preparations are essentially inert with respect to the altered protein. Furthermore, whereas ACTH behaves as a potent competitive substrate of insulin-I^{131} degradation by the soluble enzyme preparation, the same protein is without effect on the degradation of the labeled substrate by the intact liver.

Significance to NIAMD Research - Although the liver does not appear to constitute a primary target organ for the action of insulin the substantial binding of the hormone which has been observed under conditions not complicated by degradation, i.e., at 0^{o}, may bear a significant relation to the uptake of the protein by frank target organs, e.g., muscle. In particular, the location of such binding sites on or within the liver cell would be of value in the further understanding of the mechanism of action of this hormone.

Proposed Course of Project - It is proposed to couple insulin with a fluorescent dye, such as fluorescein, and to incubate the conjugate with a suspension of intact liver cells. It is then hoped to localize the site of binding of the hormone by microscopic examination. Preliminary bioassays will be necessary to determine the effect of the coupling procedure on the biological activity of the hormone.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Mortimore, G. E., Tietze, F. and Stetten, D., Jr. Metabolism of insulin-I^{131}: Studies in isolated, perfused rat liver and hind limb preparations. Diabetes 8, 307-314 (1959).

Mortimore, G. E. and Tietze, F. Studies on the fate of insulin-I^{131} in the perfused rat liver. Metabolism 8, 479-480 (1959).

Mortimore, G. E. and Tietze, F. Studies on the mechanism of capture and degradation of insulin-I^{131} by the cyclically perfused rat liver. Ann. N. Y. Acad. Sci. 82, 329-337 (1959).

Tietze, F. Release of amino acids from the carboxyl terminus of native and modified egg-white lysozyme. Arch. Biochem. Biophys. In press.

Honors and Awards relating to this project: None.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The Mechanism of Action of Hormones

Principal Investigators: Dr. Yale J. Topper and Dr. Elizabeth S. Wa

Other Investigators: Dr. T. David Elder and Dr. Stanton Segal

Cooperating Units: Section on Metabolic Enzymes - NIAMD 32
Clinical Endocrinology Branch - NIAMD 143C

Man Years (calendar year 1959):
Total: 3
Professional: 2-1/2
Other: 1/2

Project Description:

Objectives - The immediate objectives of this project are 1) to determine the mechanism(s) by which progesterone, testosterone and androsterone stimulate the oxidation of D-galactose by certain mammalian tissues in vitro and 2) to determine the mechanism by which progesterone enables galactosemic subjects to metabolize galactose.

Methods Employed - 1) Determination of $C^{14}O_2$ production from galactose-1-C^{14}. 2) Analysis of pyridine nucleotide levels in tissue preparations incubated with and without steroids. 3) Determination of certain enzymic activities as influenced by steroids.

Major Findings - Since the effect of steroids on D-galactose metabolism was reported last year the following observations have been made. 1) The site of action of progesterone on galactose metabolism has been localized at the level of the UDPGal-4-epimerase reaction. 2) One mechanism by which progesterone stimulates the epimerase reaction in liver relates to the fact that the hormone lowers the level of DPNH by inhibiting aldehyde dehydrogenase reactions. 3) Kidney aldehyde dehydrogenase is also inhibited by progesterone. It has been deduced that one of the reasons galactose metabolism in kidney is normally not influenced by progesterone relates to the virtual absence of alcohol dehydrogenase from this tissue. 4) Menthol simulates progesterone in its effects on galactose metabolism in vitro and in galactosemic subjects.

Significance to NIAMD Research - Studies on hormonal control of D-galactose catabolism might be expected to shed light not only on galactosemia, but on other metabolic diseases as well.

Proposed Course of Project - More information relating to the in vitro mechanism of action of progesterone and menthol will be sought. In addition, the mechanism of action of progesterone and menthol in galactosemia will be further investigated.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards and Publications

Publications other than abstracts from this project:

Simon, E. R., Pesch, L. A. and Topper, Y. J. Localization of the steroid hormone effect on galactose metabolism. Biochem. Biophys. Resc. Comm. 1, 6-8 (1959).

Pesch, L. A., Segel, S. and Topper, Y. J. Progesterone effects on galactose metabolism in pre-pubertal patients with congenital galactosemia and in rats maintained on high galactose diets. J. Clin. Invest. In press.

Topper, Y. J. Isomerization reactions, in "The Enzymes", Second Edition, Vol. III. Academic Press, Inc., New York. In press.

Topper, Y. J. Aldose-ketose transformations, in "The Enzymes", Second Edition, Vol. III. Academic Press, Inc., New York. In press.

Topper, Y. J., Maxwell, E. S. and Pesch, L. A. On the mechanism by which progesterone stimulates galactose metabolism. Biochim. et Biophys. Acta. In press.

Honors and Awards relating to this project: None.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Studies on Oligosaccharides and Polysaccharides

Principal Investigator: Dr. Marjorie R. Stetten

Other Investigators: Dr. DeWitt Stetten, Jr. & Mr. Howard M. Katzen

Cooperating Units: None

Man Years (calendar year 1959):
Total: 3
Professional: 2-1/2
Other: 1/2

Project Description:

Objectives - To gain insight into the normal structure, synthesis and metabolism of certain polysaccharides and oligosaccharides.

Methods Employed - Glycogen is isolated from animals by acidic methods, purified and used in studies of the nature of the changes which occur on treatment with alkali under various conditions. Light scattering methods are used in following the decline in molecular weight and chemical and chromatographic methods are used for identification of the products produced.

Usual methods for the isolation, purification, characterization and kinetic studies of enzymes are used. Radioactive glucose-1-PO_4 and maltose are prepared from radioactive starch. Samples synthesized and isolated are assayed for radioactivity.

Major Findings - The rate of degradation of glycogen by alkali under various conditions has been studied. Among the principal products of such degradations a number of mono- and polysaccharinic acids have been found. The most abundant of the monosaccharinic acids has tentatively been identified as isosaccharinic acid.

The mechanism of action of a rat liver transglucosylase has been studied and the existence of a glucosyl enzyme intermediate proposed.

Significance to NIAMD Research -- Alterations and defects in the way the body metabolizes various carbohydrates have been found to be characteristic of certain nutritional states, drug actions and metabolic diseases. Any additional knowledge as to how carbohydrates are normally handled may be expected to contribute to a better understanding of the nature of these conditions and diseases.

Proposed Course of Project - Characterization of the saccharinic acids produced by the action of alkali on glycogen will be pursued.

The purification and properties of the mammalian transglucosylation enzyme will be further studied. Evidence will be sought for the transitory existence of glycosyl enzyme complexes with transglycosylating and phosphorylating enzymes and attempts to isolate such complexes will be undertaken.

In a separate program with guest worker, Dr. Nancy Cummings, a study has been undertaken of the *in vitro* metabolism and respiration of brain tissue derived from normal and uremic rats.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Stetten, M. R. Transglucosylation by a mammalian liver enzyme. J. Am. Chem. Soc. 81, 1437-1441 (1959).

Stetten, D., Jr. Gout. Perspectives Biol. Med. 2, 185-196 (1959).

Stetten, D., Jr. Symmetry, asymmetry and meso-symmetry (Editorial). Am. J. Med. 26, 161-164 (1959).

Stetten, D., Jr. Introduction to deficiency diseases, in "Textbook of Medicine" (R. L. Cecil and R. F. Loeb, Eds.), W. B. Saunders Co., Philadelphia, pp. 527-532 (1959).

Stetten, D., Jr. A current view of metabolic errors. Am. J. Med. 26, 659-661 (1959).

Stetten, D., Jr. Hormone regulation. Rev. Mod. Phys. 31, 563-569 (1959).

Stetten, D., Jr. and Hearon, J. Z. Intellectual level measured by Army classification battery and serum uric acid concentration. Science 129, 1737 (1959).

Stetten, D., Jr. Comments on the fate of and responses to insulin in the liver. Metabolism 8, 559-564 (1959).

Honors and Awards relating to this project: None.

Serial No. [illegible]
1. Biochemistry
2. Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The Biosynthesis of Thiamine.

Principal Investigator: Dr. Irwin G. Leder

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1959):
Total: 2
Professional: 1
Other: 1

Project Description:

Objectives - To study the mechanism of synthesis of the vitamin thiamine.

Methods Employed - The enzymes which catalyze sequential steps in the synthesis of thiamine will be isolated from crude extracts of bakers' yeast by classical fractionation procedures and by column adsorption and elution techniques. The enzymatically synthesized vitamin and precursor compounds will be tested biologically with various mutants of neurospora and E. coli. Intermediates formed by purified enzyme systems will be isolated and studied by chromatographic, electrophoretic and spectrophotometric techniques.

Major Findings - The synthesis of thiamine from its two constituent moieties, 2-methyl 4-amino 5-hydroxymethyl pyrimidine and 4-methyl 5-(β hydroxyethyl) thiazole, involves the initial formation of thiamine monophosphate rather than the free vitamin. The synthesis requires three enzymatic steps: The phosphorylation of the pyrimidine to the corresponding pyrimidine pyrophosphate; the phosphorylation of the thiazole to thiazole monophosphate; the condensation of these derivatives to form thiamine monophosphate with the elimination of pyrophosphoric acid. The phosphorylated substrates have been synthesized and the "condensing" enzyme purified approximately 100-fold.

Significance to NIAMD Research - Cyclic compounds containing sulfur are represented by such diverse compounds as biotin, penicillin and thiamine. It is hoped that this study will contribute to our understanding of the synthesis and metabolism of compounds of nutritional and medicinal importance in man and in microorganisms.

Proposed Course of Project - The properties of the "condensing" enzyme will be studied. With the aid of the separate enzymes and the phosphorylated substrates, extracts of mutant microorganisms and mammalian tissues will be examined to establish the locus of the genetic defect in thiamine synthesis. These enzymes and substrates will also provide a sensitive technique for studying thiazole ring synthesis.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Leder, I. G. The enzymatic synthesis of thiamine monophosphate. Biochem. Biophys. Resc. Comm. 1, 63-66 (1959).

Honors and Awards relating to this project: None

Serial No. NIAMD-30
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Metabolic Fate of Intracellularly Generated Reduced Di- and Tri-phosphopyridine Nucleotides.

Principal Investigator: Dr. Ben Bloom

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1959):
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives - The object of this project is to gain information concerning the metabolic fate of "various" reduced diphosphopyridine nucleotides (DPNH), and "various" reduced triphosphopyridine nucleotides (TPNH) generated intracellularly.

Methods Employed - 1) Those generally used for the specific tritium labeling of substrates or when needed, the development of same. 2) Standard techniques as in current use for *in vitro* studies involving tissue slices. 3) Analysis of various metabolic products isolated from the *in vitro* incubations for tritium content.

Major Findings - The results obtained from this project, last year, suggested the possibility of using intracellularly generated reduced diphosphopyridine nucleotide-4-T for evaluating the hydroxysteroid augmented transhydrogenase concept in a cellular system. To this end the influence of several hydroxysteroids were tested for their ability to catalyze the approach toward equilibrium of the DPN-DPNH/TPN-TPNH couple. No evidence came forth suggesting that in liver cells, hydroxysteroid dehydrogenase functioned in a transhydrogenase capacity.

Significance to NIAMD Research - The concept applied in obtaining the findings recorded above can easily be used as a generalized technique for study of alterations in the DPN-DPNH/TPN-TPNH equilibrium state in both normal and pathological cellular systems. Thus an increase has been effected in the techniques available for the furtherance of our appreciation of those diseases which comprise the category of metabolic disorders.

Proposed Course of Project - The application of the above described concept to other cellular systems wherein a hydroxysteroid augmented transhydrogenase reaction might likely be found.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Bloom, B. The intracellular occurrence of reduced diphosphopyridine nucleotide-coupled reactions in liver and kidney. J. Biol. Chem. 234, 2158-2160 (1959).

Bloom, B. The hazard of orally pipetting tritium oxide. J. Lab. Clin. Med. In press.

Bloom, B. An evaluation of hormonal augmented transhydrogenase activity in rat liver cells. J. Biol. Chem. In press.

Honors and Awards relating to this project: None

Serial No. NIAMD-31
1. Biochemistry & Metabolism
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

PART A.

Project Title:

A. Studies on Steroid Reduction

B. Mechanism of Steroid Hydroxylation

C. Mechanism of Action of Steroid Hormones

D. Studies on Oxidation of Cyclic Secondary Alcohols

E. Metabolism of Steroids by Microorganisms

Principal Investigator: Gordon M. Tomkins

Other Investigators: Joseph S. McGuire
K. L. Yielding
Vincent Hollis
Jean Curran
Elizabeth S. Maxwell
Marshall Nirenberg
Richard F. Bakemeier
Giovanna Ferro-Luzzi

Cooperating Units: A. D. Merritt, Dental
Leonard Garren, Cancer
Virginia Evans, Cancer NCI-428C

Man Years (Calendar year 1959):
Total: 6
Professional: 5
Others: 1

Project Description:

Project A

OBJECTIVES - Previous work has established that there are a number of enzymes in mammalian liver which saturate the 4-5 double bond of hormonely active steroids. Enzymes in the soluble fractions of cell had been shown to be highly specific

Part B included Yes

and to produce the 5β isomer of the reduced compound. Microsomes were found to have enzymes saturating the double bond to yield the 5α isomer. These were also TPNH-specific. Questions under investigation during the past year have been:

1. What is the mechanism of the reduction?

2. What are the characteristics of the enzymes considered as proteins?

3. What is the physiological role of these enzymes?

4. What are the genetic implications of this series of proteins?

METHODS AND MAJOR FINDINGS -

Part 1, Microsomal Enzymes - A more rapid and sensitive spectrophotometric assay has been developed for the microsomal 5α steroid reductases based on the oxidation of TPNH. Most of the experiments, however, were done with an optical method based on substrate disappearance. It has been found that flavine analogs or inhibitors do not interfere with steroid reduction, and isotope studies have indicated that a proton is added to the 4- but not the 5-position in the course of the reaction, which would be predicted if there were a direct transfer of a hydride ion from TPNH to the substrate without the intermediate action of a flavine. This is an unusual mechanism for a double bond reaction and provides direct evidence for the participation of a hydride ion and a proton in this (as well as other) pyridine nucleotide-linked reactions. Hitherto, the evidence has merely been for a direct transfer of hydrogen (without specifying the electronic form) from the pyridine nucleotide to the substrate.

In addition, it has been found that there are a number of microsomal 5α reductases; possibly, each one specific for its own substrate, in the same way as the soluble 5β series. Of considerable interest was the finding that one steroid could interfere with the reduction of another by interacting with its enzyme. This inhibition was dependent on molecular size, i.e., the inhibitor had to be of a smaller size than the substrate whose reduction it was inhibiting. This indicated that the active site of the enzyme had the same contour as the substrate and interacted with it at many points, and also that this interference of the metabolism of one hormone by another may be important physiologically. The administration of small steroid hormones such as the androgens could interfere with the metabolism of larger molecules such as the adrenal cortical hormones, and overlapping effects of the hormones might be produced in that way. The microsomal enzymes appear to be "inducible" under certain circumstances. Other investigators have reported that male rats have less enzyme than female rats. We have confirmed these observations and, in addition,

found that the sex difference is independent of either gonads or adrenals since it persists in adrenalectomized, gonadectomized rats. This may be an indication that the genetic information for the enzyme resides on the X chromosome. Other explanations for it are presently being investigated. Furthermore, thyroxin was found to cause an increase in the level of the enzyme. More recently, drugs which are known to increase the levels of other microsomal enzymes, such as phenobarbital, have been found to elevate the levels of the steroid reductases. The mechanistic and physiological implications of these findings are presently being investigated.

Part 2, Soluble Enzymes - Using the methods previously developed, based on substrate disappearance, the cofactor requirement for the soluble enzymes has been clarified. It was reported previously that some of the substrates could be reduced by DPNH as well as TPNH. This finding has now been shown to be artifactual due to the finding of small amounts of TPN to various reductases, but not to all of them. It appears, therefore, that the soluble 5β reductases are TPNH specific as are their 5α counterparts. A new purification scheme for these enzymes, using DEAE cellulose, has proved highly effective and produced evidence for more discrete 5β reductases. In addition, other enzyme sources besides rat liver have been investigated, for example, pig, calf, horse, guinea pig and human liver. In the first case, pig liver, definite evidence has been obtained that there are multiple 5β reductases and these enzymes are being purified from that source by means of ammonium sulfate fractionation and ion exchange chromatography.

Part 3, Biological Considerations - There is a series of 5α reductases in the microsome, each of which is specific for its substrate in requiring TPNH, and a similar series of 5β reductases in the soluble fraction of the cell. These findings have raised two independent questions. Is there a relation between the α enzyme corresponding to a given substrate and the β enzyme, for example, is the α enzyme a precursor of a given β enzyme, or are they derived from a common precursor? The second question of interest is, in view of the multiplicity of the steroid reductases one might consider alternates to the proposition that all liver cells make each of the reductases and that there might, in fact, be "microheterogeniety" among liver cells, where one cell would make only a limited number of these enzymes, by analogy with the Burnet concept of antibody formation in which only certain cells make certain antibodies. The answer to the first question, the relation, if any, between α and β enzymes, could be obtained only if the α enzyme, on solubilization, were converted to the β enzyme, or if a genetic experiment were possible in which independent deletion of genes for the α and β enzyme could be obtained. To date, although many attempts have been made, the α enzymes have not been solubilized. Genetic experiments with mammalian liver are, of course, impossible. One approach has been to examine hepatomas which contain both α and β enzymes to see

whether deletions of an α enzyme results in loss of the corresponding β enzyme. In one case no relation between missing α and missing β enzymes was found, which suggests that at least precursor product relation between the two does not hold. In answer to the second question, whether all cells are able to make all the enzymes, two approaches have been made. The first of these is to develop specific micromethods for the determination of the enzymes in a single cell. Progress has been made toward developing methods to determine either the oxidized pyridine nucleotide produced, or substrate disappearance based on fluorescence. The second approach has been to examine tissue cultures derived from a single cell to see what their enzyme complement is. In various cases this has been done and, in fact, a limited number of enzymes has been found in tissue cultures derived from single cell. This approach has not been exploited to the fullest yet and experiments are to be continued on it in the future.

Project B.

OBJECTIVES - The method by which molecular oxygen is cleaved and one of the atoms inserted into the steroid nucleus to produce the hydroxylation reaction is unknown, although this reaction is the primary biosynthetic reaction in the synthesis of the steroid hormones as well as many other important biological compounds.

METHODS AND FINDINGS - Additional studies with mammalian systems has progressed although those studies using microbial systems have been dropped due to extreme lability of the enzymes involved. Mammalian experiments have confirmed the fact that three enzymes and TPNH are involved as well as the heat stable cofactor. Some indication of the nature of this cofactor has been obtained recently. It seems to be a carbohydrate, possibly a phosphorylated hexose. One of the enzymes involved in hydroxylation may be able to convert glucose-6-phosphate to the cofactor. In the presence of large amounts of boiled liver extract, one of the enzymes can be eliminated from the reaction mixture. However, with G-6-P as the precursor of the cofactor, this enzyme must be present.

Project C.

OBJECTIVES - The steroid hormones are potent biological reagents and an understanding of their action at a molecular level is essential for an understanding of physiological control mechanisms in the cell. To date little specific information is available and theoretical controversies rage over even such mechanisms as have been presented.

METHODS AND MAJOR FINDINGS - We ave found that numerous steroid hormones inhibit DPNH cytochrome c reductase from many sources, both mammalian and microbial, as well as from neoplastic tissues. The site of this inhibition has been localized to a step between the flavoprotein and cytochrome b, the same step affected by amytol. Its inhibition is competitively reversed by α-tocopherol and other lipids. In some tissues a steroid insensitive pathway of electron transport has been discovered where electrons are transferred directly from flavoprotein to cytochrome c, circumventing cytochrome b. Methods involved in this study have been primarily spectrophotometric assays of reduced pyridine nucleotide oxidation or cytochrome oxidation reduction. In addition, conventional Warburg manometry and respirometric measurements with the Clark oxygen electrode have been used. Since this reaction is the main pathway of electron transport beyond the substrate level, interference of it by steroid hormones is of obvious physiological importance. However, the inhibition is so general, not only in terms of tissues affected, but also in terms of what steroids are effective that it is difficult at the present time to see how the specific effect attributed to steroid hormones can be as a result of this inhibition. Studies have been undertaken, however, to determine whether, in fact, specific effects can be observed on the basis of inhibition of this sort. One of the forms of steroid inhibition of electron transport that might be reflected in the physiological effect is the carcinostatic effect of steroids. To this end steroid-sensitive and steroid-resistant tumor lines have been developed and the amount and sensitivity of the DPNH cytochrome c reductase enzyme has been determined. Other studies have been continued on the use of steroids as TPNH oxidizing agents in intracellular economy. As previously reported, various steroids which can provide TPN through double bond reduction, stimulate the oxidation of glucose-6-phosphate in this way. Net accumulation of TPN in the presence of different steroid hormones has been shown using isolated intact liver cells so that this metabolic effect may, in fact, play a role in steroid metabolism.

Project D.

OBJECTIVES - Previously it has been shown that crystalline horse liver alcohol dehydrogenase could oxidize cyclic secondary alcohol. Since these compounds are structurally related to steroids, a study of the interaction of the enzyme with these compounds was undertaken. This has been pursued somewhat during the course of the past year.

METHODS AND FINDINGS - Findings of further interest have been that the axial hydroxyl is preferentially oxidized by DPN with alcohol dehydrogenase. This conclusion was derived from the fact that *cis* 4 tertiary butyl cyclohexanol is

oxidized by the enzyme in DPN, but the trans isomer is not. Since the tertiary butyl group is sufficiently bulky to be fixed in the equatorial conformation, the cis hydroxyl group is necessarily axial and the trans necessarily equatorial. The cis hydroxy compound is oxidized. This is an interesting situation in view of the fact that the liver 3α hydroxysteroid dehydrogenase preferentially oxidizes the equatorial hydroxyl as one might expect, since it is the more unhindered project alcohol group.

Project E.

OBJECTIVES - Microorganisms are able to metabolize steroids often in a dramatic way, although the role of steroids in the metabolism of microorganisms is thoroughly unknown. Recently, as described in another project report by E. S. Maxwell, we have isolated a mutant yeast which is resistant to the antibiotic effect of steroids. Some of the metabolic transformations of the steroids by the yeast have been investigated.

METHODS AND MAJOR FINDINGS - From both mutant and wild type Saccharomyces fragilis, a metabolite of 4-androstene-3,17-dione was recovered which was considerably less polar by paper chromatography, which was rendered more polar by alkaline hydrolysis. Chromic acid oxidation of the hydrolyzed compound yielded the substrate, 4-androstene-3,17-dione. These facts can be reconciled with the formulation that 4-androstene 3,17-dione is first reduced to testosterone following which the testosterone is conjugated to form an acetylated compound at 17 testosterone acetate. This compound has never before been found in natural sources and it is therefore of considerable interest. The identity of the isolated conjugate has been further confirmed by first hydrolysis and activation of the acetate moiety with acetokinase, following which the hydroxamic acid was chromatographed. Infrared studies of the intact conjugate also indicated that it was testosterone acetate.

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Tomkins, G. Studies on the Mechanism of Steroid Hydroxylation. Colloquim on Oxygenating Enzymes, 4th Int. Congress of Biochemistry, Vienna.

McGuire, Joseph S. and Tomkins, Gordon M. The Effects of Thyroid Administration on the Enzymic Reduction of Δ^4-3-keto steroids. J. Biol. Chem., 234, 791 (1959).

Tomkins, Gordon M. Enzymatic Metabolism of Corticosteroids. Ann. New York Acad. of Sci., 82, 836 (1959).

Merritt, A. Donald and Tomkins, Gordon M. Reversible Oxidation of Cyclic Secondary Alcohols by Liver Alcohol Dehydrogenases. J. Biol. Chem., 234, 2778 (1959).

Yielding, K. Lemone and Tomkins, Gordon M. Inhibition of Enzymic Oxidation of DPNH by Steroid Hormones. Proc. Nat. Acad. Sci. (In press).

Yielding, K. Lemone and Tomkins, Gordon M. An Effect of Enzymic Reduction of Steroids on Triphosphopyridine Nucleotide-Dependent Glucose-6-phosphate Oxidation. Biochim. et Biophys. Acta (in press).

Tomkins, Gordon M. and McGuire, Joseph S. The Effect of Thyroid Hormones on Adrenal Steroid Metabolism. N. Y. Acad of Sci. (In press).

McGuire, Joseph S. and Tomkins, Gordon M. The Multiplicity and Specificity of Δ^4-3-ketosteroid 11β-Hydrogenases (59). Arch. Biochem. & Biophys. 82, 477 (1959).

Tomkins, Gordon M. and McGuire, Joseph S. The Adrenogenital Syndrome. In J. B. Stanbury, J. B. Wyngaarden and D. S. Fredrickson (Editors) The Metabolic Basis for Inherited Disease, McGraw-Hill, New York (in press).

McGuire, J. S. and Tomkins, G. M. The Heterogeneity of Δ^4-3-keto-steroid reductases (59). J. Biol. Chem. (in press).

PHS-NIH
Individual Project Report
Calendar Year 1959

PART A.

Project Title:

I. Galactose Metabolism

A. UDPgalactose-4-epimerase from yeast.

B. A Steroid Sensitive Aldehyde Dehydrogenase

C. An Assay for Galactose-1-P in Human Erythrocytes

II. The Antibiotic Effect of Steroid Hormones on Yeast and the Isolation of a Resistant Mutant

Principal Investigator: E. Maxwell

Other Investigators: Gordon Tomkins, Joseph McGuire, Leroy Pesch, Yale Topper, H. N. Kirkman

Cooperating Units:

Man Years (Calendar Year 1959)

Total:	3
Professional:	2
Other:	1

Project Description:

I. Galactose Metabolism

OBJECTIVES -

A. Previous studies carried out in collaboration with Dr. H. M. Kalckar and Dr. Huguette Szulmajster demonstrated that UDPgalactose-4-epimerase purified from yeast differs in several respects from the same enzyme from calf liver. The enzyme from liver requires exogenous DPN and is inhibited by DPNH. In contrast the enzyme from galactose-adapted yeast is fully active without the addition of DPN and is not inhibited by DPNH. The enzyme from yeast contains a tightly bound substance which fluoresces with the characteristics of DPNH. Treatment with p-chloromercuribenzoate brings about a disappearance of fluorescence and a loss of enzymic activity. Activity but not fluorescence can be restored with DPN

Part B included Yes

plus cysteine but not with cysteine alone. These findings suggested that the yeast enzyme contains protein-bound pyridine nucleotide. Studies were, therefore, undertaken to identify the protein-bound material and to investigate further the mechanism of the enzymic interconversion of UDPgalactose and UDPglucose.

B. Previous studies carried out by Dr. Yale Topper, Dr. Leroy Pesch and Dr. Ernest Simon demonstrated that the oxidation of galactose by the soluble fraction of rabbit liver, as measured by the liberation of C-1 as CO_2, is stimulated by certain steroid hormones and that the interconversion of UDP galactose and UDPglucose is a limiting step in the reaction sequence. In collaboration with Dr. Topper and Dr. Pesch, the mechanism of the steroid stimulation of galactose metabolism has been investigated.

C. In collaboration with Dr. Neil Kirkman, the development of a method for determining galactose-1-phosphate in red blood cells was undertaken.

MAJOR FINDINGS AND PROPOSED COURSE

A. An improved method for the purification of UDPgalactose-4-epimerase from galactose-adapted yeast was devised. The purified enzyme was shown by three independent methods to contain protein-bound DPN. About 0.3 μmole of DPN per 100 mg. protein was present in the most purified preparation. Using DEAE cellulose chromatography a correlation was demonstrated between bound DPN, enzymic activity and fluorescence at 450 mμ. Whether or not the fluorescence is due to bound DPN is not yet certain, but it seems clear that the mechanism of the yeast enzyme is similar to that of the same enzyme from liver. The difference in response to exogenous DPN can be explained by the presence of tightly bound DPN in the purified enzyme from yeast. The yeast enzyme, like the enzyme from liver, failed to incorporate tritium into the hexose nucleotide from either tritiated water or DPNH labeled with tritium in the para position. The detailed mechanism of the reaction and the role of DPN thus remains to be determined.

B. In collaboration with Dr. Pesch and Dr. Topper, it was found that progesterone and certain other steroid hormones bring about a decrease in the rate of reduction of DPN in systems previously employed by these investigators for studies on the stimulation of galactose oxidation by steroids. Since UDPgalactose-4-epimerase in liver requires DPN and is inhibited by DPNH, a decreased rate of reduction of DPN would be expected to result in stimulation of the conversion of UDPgalactose to UDPglucose, a step previously shown to be limiting in the reaction sequence leading to CO_2 formation from C-1 of galactose. The mechanism by which

progesterone decreases the rate of DPN reduction has been investigated and is now at least partially understood. The 40-60 per cent saturated $(NH_4)_2SO_4$ precipitate from the soluble fraction of rabbit liver contains DPN-specific aldehyde dehydrogenase activity which is 40-75 per cent inhibited by 10^{-5} M progesterone. The system is active with a number of aldehydes including, acetaldehyde, propionaldehyde, glycolaldehyde, succinic semialdehyde and glyceraldehyde. When coupled with alcohol dehydrogenase, the reduction of DPN by alcohols, such as propylene glycol, can also be shown to be inhibited by progesterone. The steroid is not acting stoichiometrically as an electron acceptor since the difference in DPNH concentration in systems with and without added steroid is as much as 60 times the concentration of steroid present. The 0-40 per cent saturated $(NH_4)_2SO_4$ precipitate also contains aldehyde dehydrogenase activity but this activity is unaffected by progesterone. Aldehyde dehydrogenase purified from calf liver according to the method of Racker is similarly unaffected.

The steroid-sensitive aldehyde dehydrogenase is being purified from rabbit liver. The properties of the purified enzyme, or enzymes, will be investigated in detail. Aldehyde and steroid specificity, as well as tissue and species distribution, will be investigated and attempts will be made to evaluate the physiological significance of the reaction.

C. In collaboration with Dr. Neil Kirkman, a sensitive, highly specific and comparatively simple assay for galactose-1-P in erythrocytes has been devised. Such an assay applied to galactosemic patients should be of value to physicians in determining the efficiency of galactose-free diets or for detecting divergence from such prescribed diets.

II. The Effect of Steroid Hormones on the Growth of Yeast.

OBJECTIVES - Studies in other laboratories indicate that the growth of a number of Gram-positive bacteria is inhibited by a variety of steroid hormones. Although certain organisms, including yeast, are capable of synthesizing steroids from acetate and of metabolizing steroids by reactions similar to those occurring in mammalian steroid hormone biosynthesis, the physiological significance of steroids in microorganism is not known. The present studies were undertaken in collaboration with Dr. Joseph McGuire and Dr. Gordon Tomkins in an attempt to gain some information about the role of steroids in Saccharomyces fragilis.

MAJOR FINDINGS - The growth of wild-type S. fragilis is almost completely inhibited by 0.13 mg/ml. of 4-androstene-3,17-dione, 1-androstene-3,17-dione, androstane-3,17-dione, deoxycorticosterone and progesterone. Other closely related steroids either had no

effect on growth or inhibited to a much less extent. One of the inhibitory steroids, 4-androstene-3,17-dione, was shown to be lethal to growing cells of this species of yeast. Resting cells were much more resistant to the steroid.

Several mutant strains of S. fragilis were isolated which were relatively resistant to steroids. Attempts were made to discover the basis of their resistance. No qualitative difference in the metabolism of 4-androstene-3,17-dione was observed in the wild-type and resistant strains. Whole cells of both strains convert the added steroid to a previously undescribed metabolite which has been identified by Dr. McGuire as testosterone acetate. The uptake of C^{14} labeled 4-androstene-3,17-dione into growing cells of the resistant mutant was about half as fast during the log phase of growth as was that at the same phase into wild-type cells. In both cases the rate of uptake was slow during rapid growth. As the stationary phase approached, the rate of uptake increased and became the same in resistant and sensitive strains. Whether or not the slower rate of steroid uptake into mutant cells is sufficient to account for their resistance is not yet known. Further studies will be instigated to delineate the mechanism of steroid resistance in the mutants.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part E. Honors, Awards, and Publications

Publications other than abstracts from this project:

Anderson, E. P., Maxwell, E. S. and Burton, R. M. The Enzymatic Synthesis of C^{14} labeled UDP glucose, UDP galactose and Galactose-1-phosphate. J. Am. Chem. Soc. (in press).

Maxwell, E. S., and Szulmajster, H. de Robichon. The Purification of UDPgalactose-4-epimerase from Yeast and the Identification of Protein-bound Diphosphopyridine Nucleotide. J. Biol. Chem. (in press).

Maxwell, E. S. Enzymic Epimerization. Vol. III The Enzymes (in press).

Kirkman, H. N. and Maxwell, E. S. Enzymatic Estimation of Erythrocytic Galactose-1-phosphate. J. Lab. and Clin. Med. (in press).

Maxwell, E. S., McGuire, J. S. and Tomkins, G. M. The Antibiotic Effect of Steroids on Saccharomyces fragilis and the Isolation of a Resistant Mutant. J. Bact. (In press).

Topper, Y. J., Maxwell, E. S. and Pesch, L., On the Mechanism by which Progesterone Stimulates Galactose Metabolism. Biochim. et Biophys. Acta (in press).

PHS-NIH
Individual Project Report
Calendar Year 1959

PART A.

Project Title:

1. γ-Hydroxybutyric Acid Catabolism

   a. The pathway of γ-hydroxybutyric acid metabolism

   b. Shared genetic information - A test case

   c. The mechanism of succinic semialdehyde oxidation

2. The role of the inducer in penicillinase induction

Principal Investigator: Marshall Nirenberg

Other Investigators:

Project 1. a, b, c. Dr. William Jakoby

Project 2. Dr. Gordon M. Tomkins

Cooperating Units: None

Man Years (Calendar year 1959)

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

Project Description:

OBJECTIVES - 1a. To determine the enzymatic steps involved in the oxidation of γ-hydroxybutyric acid by Pseudomonas fluorescens.

1b. To determine whether one cystron contains the information necessary for the synthesis of a protein subunit which may be an integral part of two or more enzymes. The γ-hydroxybutyric and β-hydroxypropionate dehydrogenase systems will be investigated.

1c. To obtain information about the sites of substrate attachment of TPN-succinic semialdehyde dehydrogenase in order to devise an approximation of the mechanism of aldehyde oxidation.

Part B included Yes

METHODS AND MAJOR FINDINGS

1a - γ-Hydroxybutyric acid dehydrogenase and two different succinic semialdehyde dehydrogenases have been purified from extracts of Pseudomonas species and the properties of these enzymes have been described. γ-Hydroxybutyric acid dehydrogenase is specific for γ-hydroxybuterate oxidation and forms succinic aldehyde as the product. Diphosphopyridine nucleotide is required for the reaction. Both succinic semialdehyde dehydrogenases are specific for succinic semialdehyde oxidation and form succinic acid as the product. One enzyme has a high affinity for diphosphopyridine nucleotide, the other for triphosphopyridine nucleotide. The differences between the enzymes as well as their possible relationships have been investigated.

1b. The induction of the enzymes in the pathway of γ-hydroxybutyric acid metabolism was investigated. A β-hydroxypropionic acid dehydrogenase was formed by these microorganisms when they were grown upon β-hydroxypropionic acid as the sole carbon source. The appearance of both γ-hydroxybutyric acid dehydrogenase and β-hydroxypropionic acid dehydrogenase were dependent upon the growth phase of the culture. γ-Hydroxybutyric acid was found to induce the formation of γ-hydroxybutyric acid dehydrogenase at low inducer concentrations. Higher concentrations of γ-hydroxybutyric acid were highly effective inducers not only of γ-hydroxybutyric acid dehydrogenase but also of β-hydroxypropionic acid dehydrogenase. The relationships between the inductions of similar enzymes in different metabolic pathways by the same inducer were investigated further. No evidence of shared genetic information between the two closely related enzymes was found.

1c. The effect of trypsin upon the TPN succinic semialdehyde dehydrogenase was investigated. When TPN combined with the enzyme, an intramolecular rearrangement of the enzyme occurred which exposed a bond labile to trypsin activity. Enzymatic activity could then be rapidly destroyed by trypsin. This phenomenon was utilized to study the half reactions involved in succinic semialdehyde oxidation. The enzyme was inhibited by arsenite which suggested that two closely juxtaposed sulfhydryl groups were present. The pyridine nucleotide cofactor did not combine with the sulfhydryl groups, instead competition between arsenite and the aldehyde substrate for at least one of the two closely juxtaposed sulfhydryl groups occurred. On the basis of these findings a mechanism of aldehyde oxidation was proposed.

OBJECTIVES - 2 - To investigate various parameters of Pollack's penicillinase system with the hope of gaining some knowledge of the role of the inducer during enzyme induction.

METHODS AND MAJOR FINDINGS

Possible roles of the inducer are now being studied in penicillinase induction in B. cereus. Induction of penicillinase is being studied in protoplasts and protoplast lysates. Highly sensitive methods of assaying penicillinase have been developed.

Part B. Honors, Awards and Publications

Nirenberg, M. A Biochemical Characteristic of Ascites Tumors. J. Biol. Chem. (in press).

Nirenberg, M. and Jakoby, W. The enzymatic Utilization of γ-Hydroxybutyric Acid. J. Biol. Chem. (in press).

Nirenberg, M. W. and Jakoby, W. B. On the Sites of Attachment and Reaction of Aldehyde Dehydrogenases. Proc. Nat. Acad. Sci. (in press).

Serial No. NIAMD - 34
1. Biochemistry & Metabolism
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

PART A.

Project Title:

Structural study of nucleotides, polynucleotides, and nucleic acids by means of infrared spectra in D_2O solution.

Principal Investigator: H. Todd Miles

Other Investigators: None

Cooperating Units: None

Man Years (Calendar year 1959)
Total: 1
Professional: 1
Other: 0

Project Description

OBJECTIVES - This project has been primarily concerned with a study of the tautomeric forms of the nucleotide components of nucleic acids and with the application of the information obtained to the structures of nucleic acids.

METHODS EMPLOYED - The infrared spectra of nucleotides, polynucleotides, and nucleic acids have been observed in D_2O solution. A number of model compounds have been synthesized to permit the spectra to be interpreted in structural terms.

MAJOR FINDINGS - During the past year it has been found that polyinosinic acid definitely exists in the keto form in aqueous solution and polycytidylic acid probably in the amino form, and that these tautomeric structures are maintained in the helical interaction product formed by mixing the polymers. In addition it has been proposed that the changes which occur in the spectra upon mixing the polynucleotides may be explained largely by the reduction in dielectric constant caused when the close-packed helices are formed with consequent exclusion of water from the surfaces of the heterocyclic rings. The changes in spectra of DNA upon denaturation obtained by other workers may very well have the same explanation.

Part B included Yes

The helical interaction products of the polynucleotides have been found to have essentially the same stability in D_2O as in H_2O solution, demonstrating the applicability of the results obtained in the former solvent to the latter as well.

SIGNIFICANCE TO BIOMEDICAL RESEARCH - The question of tautomeric forms of the nucleotides is fundamental to the structures of the nucleic acids since the hydrogen bonding schemes that hold the nucleic acid helices together are determined by this structural feature of the component nucleotides. A related point of biological interest is the proposal of Watson and Crick that the chemical mechanism of mutation involves formation of the less stable tautomeric form in a polynucleotide chain with consequent pairing with the "wrong" base in a DNA molecule.

Part B. Honors, Awards and Publications

Miles, H. T. A Proposed Interpretation of Infrared Spectral Changes Occurring upon the Interaction of Polynucleotides. Nature 183, 1814 (1959).

Miles, H. T. Infrared Spectra and Tautomeric Structure of Polyinosinic and Polycytidylic Acids in D_2O Solution. Biochim. et Biophys. Acta 34, 274 (1959).

Miles, H. Todd, Smyrniotis, P. Z., and Stadtman, E. R. Bacterial Degradation Products of Riboflavin. III. Isolation, Structure Determination and Biological Transformations of 1-Ribityl-2,3-diketo-1,2,3,4-tetrahydro-6,7-dimethylquinoxaline, J. Am. Chem. Soc., 81, 1946 (1959).

Serial No. NIAMD- 35

1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Metabolism and Biosynthesis of Catechol Amines

Principal Investigator (at NIAMD): Bernhard Witkop

Other Investigators: Siro Senoh (left 3/17/59), John Daly, Y. Kaneoka (V.S. arrived 8/31/59)

Cooperating Units: S. Udenfriend and C. R. Creveling, NHI, Serial No. NHI-216 Dr. Sydney Archer, Sterling-Winthrop Research Institute

Man Years (calendar year 1959):
Total: 2/3
Professional: 2/3
Other: 0

Project Description:

Synthesis of Novel Metabolites of Dopamine, Norepinephrine, Adrenaline and Other Catechols of Physiological Importance. Clarification of the Biosynthesis of Norepinephrine.

Objectives: To establish metabolic parameters for important endogenous hormones, to characterize new catechol metabolites, to find labile transformation products of dopamine and precursors of norepinephrine by elucidating the mechanism of its formation.

Methods Employed: Cross labeling of dopamine with tritium and C^{14} was used to follow the chemical and enzymatic transformations including the conversion to noradrenaline.

Major Findings: The addition of nucleophilic reagents such as water or methanol to (N-acylated) dopaminequinones produced noradrenaline and 6-hydroxydopamine (2,4,5-trihydroxyphenylethylamine) in a ratio of 10,000:1. 2,4,5-Trihydroxyphenylethylamine is easily formed by autoxidation from solutions of dopamine on standing, with boiled tissue or in the ascorbic acid-versene system. This new autoxidation product of dopamine is chromatographically indistinguishable from noradrenaline. Its discovery was made possible only by cross-labeling technique whereby it was found that the "noradrenaline" fraction had

not lost any significant tritium activity. The same technique showed that tissue from selected regions of the brain, such as hypothalamus and caudate nucleus, convert dopamine to authentic noradrenaline in yields up to 4%.

A number of new aminochromes and tetrasubstituted indoles have been prepared from derivatives of dopaminequinone and the mechanism of these transformations has been followed by tritium labeling. It remains to be seen whether these new aminochromes or some of their derivatives are centrally active. Such activity has been claimed without sufficient support for adrenochrome and adrenolutin.

Since it has recently been postulated that dopamine may be a new hormone in certain tissues, it is of interest to note the excretion of 6-hydroxydopamine after administration of dopamine to animals.

Collaborative efforts with Sterling-Winthrop aim at the synthesis of amino acid precursors capable of penetrating the blood-brain barrier and of the release of active amines such as adrenaline, normetanephrine etc. in the brain. It has been noted in the National Institute of Mental Health that these biogenic amines when labeled and administered to animals did not reach the brain.

Significance to bio-medical research and the program of the Institute: The metabolic fate of peripherally and centrally active biogenic amines is a key problem in modern neurochemistry and psychopharmacology.

Proposed Course of Project: In analogy to the formation of 6-hydroxydopamine (2,4,5-trihydroxyphenylethylamine) one may expect the occurrence of 6-hydroxy-(nor)epinephrine by a similar mechanism. Judging from previous experience with 6-hydroxydopamine, such a hydroxyadrenaline analog would have to be synthesized first and known in all its properties before attempts could be made to prove its presence as a metabolite. Such synthetic studies are planned.

Part B included: Yes

Part B. Honors, Awards, and Publications

Dr. Witkop received the 1958 Hillebrand Award of the Washington Section of the American Chemical Society for outstanding contributions to the structure and oxidation mechanisms of natural products and intermediary metabolites.

Publications other than abstracts from this project:

Senoh, S., Witkop, B., Creveling, C. R. and Udenfriend, S.: Oxidation Mechanisms of Catecholamines and the Biogenesis of Noradrenaline. Fourth International Congress of Biochemistry 13, 176-188, 1959.

Senoh, S., Witkop, B.: Formation and Rearrangements of Aminochromes from a New Metabolite of Dopamine and Some of its Derivatives. J. Am. Chem. Soc. 81, 6231-6235, 1959.

Senoh, S., Witkop, B.: Non-Enzymatic Conversions of Dopamine to Norepinephrine and Trihydroxyphenethylamines. J. Am. Chem. Soc. 81, 6222-6231, 1959.

Senoh, S., Creveling, C. R., Udenfriend, S. and Witkop, B.: Chemical, Enzymatic and Metabolic Studies on the Mechanism of Oxidation of Dopamine. J. Am. Chem. Soc. 81, 6236-6240, 1959.

Kny, H. and Witkop, B.: Chemical and Enzymatic Studies of the Labile Metabolite 4(5H)-Imidazolone-5-acetic Acid. J. Am. Chem. Soc. 6245-6251, 1959.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Selective Cleavages of Peptide Bonds by Chemical Agents

Principal Investigator: Bernhard Witkop

Other Investigators: W. B. Lawson, Erhard Gross, L. K. Ramachandran

Cooperating Units: T. Visvanatha, LFT, L. C. Craig, The Rockefeller Institute for Medical Research

Man Years (calendar year 1959):
Total: 2
Professional: 2
Other: 0

Project Description:

Objectives: 1) To investigate and apply methods for the selective cleavage of peptide bonds. 2) To selectively modify or cleave peptides, proteins and enzymes in order to correlate structural elements with physiological or enzymatic activity.

Methods Employed: Special organic oxidizing agents, such as N-bromosuccinimide, N-bromoacetamide, sodium periodate and others, are capable of selectively attacking, e.g., tryptophan residues in peptides and proteins. In situ observation of the changes in absorption by differential ultraviolet spectrophotometry in a self-recording instrument is used to follow this reaction. DNP and Stein and Moore techniques, paper chromatography and electrophoresis serve for the identification of cleavage products.

Major Findings: The well-known photo-oxidation of tryptophan-containing proteins is accompanied by characteristic shifts in the ultraviolet spectrum to shorter wavelengths. Similar shifts are also produced by the controlled oxidation of, e.g., N-Cbz-tryptophan, poly-tryptophan, gramicidin, lysozyme, chymotrypsin, etc., with selective oxidants, such as N-bromosuccinimide in water. The reaction which was followed in situ by differential UV-spectrophotometry proceeded smoothly in $\sim 10^{-5}$ molar solution. After the rapid consumption of approximately 1.5 ml. of NBS per mole of tryptophan the indole chromophor had disappeared. Volhard titration of the reaction mixture showed the presence of only $\sim$80% bromide ion pointing to nuclear bromination accompanying oxidation of the indole ring to derivatives of oxindole. Model studies with skatol, indole-β-propionic acid and

N-benzoyl-tryptophan showed that substitution, oxidation and group participation merged in the bromination of indoles.

The structure of the dibromoskatole, resulting from the action of N-bromophthalimide on skatole in benzene, has been proved to be 2,6-dibromoskatole by acid hydrolysis to 6-bromo-3-methyloxindole, an isomer of the bromination product of 3-methyloxindole, and by oxidative degradation of 2-acetamino-4-bromobenzoic acid. Electrophilic substitution of indoles in the 6-position has been shown, in the case of 2-phenylskatole, to proceed via an unstable yellow perbromide intermediate, rearranging rapidly to the 6-bromo compound. In aqueous media, intramolecular participation of the carboxyl group of indole-3-propionic acid, possibly by displacement on a bromonium intermediate, has led to (5-bromo)dioxindolespirolactones which have been hydrogenolyzed to oxindole-3-propionic acid.

This neighboring group effect of a potentially nucleophilic amide imidol group in an indole β-side chain was utilized for the cleavage of the N-peptide bond adjacent to tryptophan. Whereas NBS treatment of N-Cbz-tryptophyl-glycine gave free glycine, the isomeric N-Cbz-glycyltryptophan under these conditions did not liberate an amino acid. The general usefulness of the method was demonstrated with glucagon, the crystalline hyperglycemic-glycogenolytic peptide from pancreas, containing only one tryptophan among 29 amino acids. N-Bromosuccinimide leads to the liberation of a major new ninhydrin-positive peptide, giving positive platinic chloride reaction for methionine and negative reactions for histidine and arginine. Its hydrolysis yielded aspartic acid, threonine, methionine and leucine. This tetrapeptide, which arises from the C-terminal sequence TRY-LEU-MET-ASP-THR, has been obtained by the action of chymotrypsin and trypsin on glucagon. However, the cleavage of glucagon by N-bromosuccinimide is more rapid (<1 min.) and more selective than that by any known peptidase.

The reactions of trypsin, trypsinogen, acetyltrypsinogen, and an enzymatically active fragment of trypsinogen with N-bromosuccinimide have been explored. Under the conditions used, the reagent selectively oxidized the tryptophan residues without significant cleavage of tryptophyl peptide bonds. The marked difference in reactivity of tryptophan in trypsin and trypsinogen is ascribed to differences in their secondary or tertiary structure. Enzymatic inactivation (trypsin) or loss of activatability (trypsinogen) was studied as a function of the oxidative modification of tryptophan. Such partially inactivated enzyme preparations still had their DFP phosphorylation sites intact. At least one tryptophan residue may be needed for activity. This demonstrates that an intact phosphorylation site per se is not sufficient for enzymatic activity.

The application of the N-bromosuccinimide (NBS) cleavage to proteins under specified conditions releases new N-terminal residues. Bond cleavages generally average 20-40% and the number of new N-terminals formed corresponds to the number of tryptophans in the molecule. The results indicate the presence of Try-Lys and Try-Ala bonds in tobacco mosaic virus (TMV) protein, of a Try-Ala bond in the I-peptide from TMV protein, of a Try-Ala bond in human serum albumin, and of Try-Gly and Try-Ser bonds in bovine serum albumin. Lysozyme which contains seven tryptophans is cleaved by the reagent with much lower yields.

Significance to bio-medical research and the program of the Institute: There is a great need for mild and selective methods for the controlled and systematic degradation of proteins. These chemical "peptidases" in many ways promise to be superior to all known enzymes customarily used for breakdown and structural investigation of proteins.

Proposed Course of Project: Dr. Gross has spent some time in Dr. L. C. Craig's laboratory at the Rockefeller Institute to find conditions for the cleavage of a number of cyclic antibiotic peptides. Active work is directed toward elucidation of gramicidin A. New cleavage reagents and conditions are being investigated.

Part B included: Yes

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Ramachandran, L. K., and Witkop, B.: Selective Cleavage of C-Tryptophyl Peptide Bonds in Proteins and Peptides. J. Am. Chem. Soc. 81: 4028-4032, 1959.

Serial No. NIAMD-37

1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Studies on Substrates and Inhibitors of Cholinesterase and on the Chemistry of Neuro-Muscular Blocking Agents

Principal Investigator (at NIH): Bernhard Witkop

Other Investigators: H. Kny (Left NIH 11/2/59), J. W. Daly

Cooperating Units: S. Friess, R. C. Durant, Naval Medical Center

Man Years (Calendar year 1959):
Total: 1/3
Professional: 1/3
Other: 0

Project Description:

Objectives: To establish a role and possibly a use for derivatives of those mono- and diaminocheinitols that occur as building stones of antibiotics; to explore the steric limitations and requirements for (polyfunctional) substrates of cholinesterase; to correlate neuromuscular blocking activity with the structure of cyclic analogs of dimethylaminoethanol.

Major Findings: In order to investigate the labilization of ester bonds in the acetates of tertiary and quaternary 2-deoxy-2-dimethylamino-myo- and scyllo-inositol and of O-acetates in the streptamine series, the synthesis of analogous compounds in the N,N-tetramethyldeoxystreptamine series was carried out. An extremely labile tri-O-acetate was obtained, the hydrolytic activity of which approached that of esters as labile as p-nitrophenyl acetates. Two diacetates were obtained, one of which was quite labile while the other was fairly unreactive. Nuclear magnetic resonance investigation suggested that the reactive diacetate was the symmetrical 4,6-O-diacetyl derivative. The quaternary salts of these esters were prepared and are being investigated. It will be of great interest if the labilisation of the esters in these model compounds can be correlated with the overall conformation of the molecule.

The anticholinesterase activity of synthetic D,L-muscarine and 9 further derivatives was determined with a highly purified enzyme

preparation from electric eel tissue by a constant pH titration technique used previously (S. L. Friess, A. J. Patchett and B. [illegible], J. Am. Chem. Soc. 79: 459, 1957). A greater activity than by acetyl- was exhibited by trans-4,5-dihydromuscarine, allo-muscarone and O-acetylmuscarine. These enzymatic results parallel the pharmacological findings.

Significance to bio-medical research and the program of the Institute: Two major aspects of the cholinesterase problem are of special interest. 1) The mode of hydrolysis and nature of ester labilization. 2) The correlation of structure and activity in natural and synthetic inhibitors.

Proposed Course of Project: Dr. Friess is presently occupied with pharmacological and isolated nerve studies involving inhibitors of cholinesterase. It will be of special importance to construct inhibitors capable of penetrating the blood-brain barrier and of acting on brain cholinesterase directly.

Part B included: Yes

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Witkop, B., Durant, R. C. and Friess, S. L.: Acetylcholinesterase Inhibitory Activities of Muscarine and Muscarone Derivatives. Experientia 15: 300-301, 1959.

Friess, S. L., Standaert, F. G., Witkop, B., Durant, R. C. and Reber, L. J.: Some Toxicologic Properties of a New Series of Aryl Ethers Derived from Trans-2-Aminocyclohexanol. Toxicol. Applied Pharmacol. 1, 609-617, 1959.

1. Chemistry
2. Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Inhibitors of the Biosynthesis and Breakdown of 5-Hydroxytryptamine and Other Centrally Active Biogenic Amines

Principal Investigator (at NIAMD): Bernhard Witkop

Other Investigators: M. Ozaki

Cooperating Units: J. Udenfriend, H. Weissbach and B. Peterkofsky, Laboratory of Chemical Pharmacology, NHI
Serial No. NHI- 246

Man Years (Calendar year 1959):
Total: 2
Professional: 1
Other: 1

Project Description:

Project: Synthesis of compounds which inhibit 5-hydroxytryptophan decarboxylase, dopa-decarboxylase and monoamine oxidase (MAO).

Objectives: The inhibition of the enzyme which converts 5-OH-tryptophan or 5-hydroxyphenylalanine would prevent the formation of serotonin or dopamine and other catecholamines and hence possible applicability in diseases characterized by overproduction of serotonin and catecholamines such as Hartup carcinoid syndrome, pheochromocytoma tumor and chronic hypertension. The inhibition of MAO in peripheral or central locations is known to result in marked pharmacological effects. The control of both of these processes is highly desirable.

Methods Employed: The technique for assaying MAO activity in vitro is described in the publication by Pletcher, Weissbach et al. Similar techniques are being used or investigated by Dr. M. Ozaki working in Dr. Udenfriend's laboratory for the decarboxylases acting on 5-hydroxytryptophan and 5-hydroxytryptophan, as well as on acetylcholine and transferase.

Major Findings: In the laboratory of Dr. Udenfriend, Dr. [illegible] has been screening over 80 compounds for inhibition of serotonin. Most tests were performed in vitro following the disappearance of serotonin, and in some cases dopamine. Some very active inhibitors

have been studied in vivo in rats, and serotonin brain levels have been determined in a number of cases. Other studies concerned the activity of monamine oxidase in various organs as a function of the species. For instance, determinations were made in brain, liver and kidney of mice, rabbits, dogs, rats, cats, guinea pigs, hamsters and toads. The effect of various monamine oxidase inhibitors was not the same in all animals: it was found that Marsilid does not act in toad liver, whereas amphetamine hydrazine (JB 516) is active.

Over 30 compounds were tested for activity as inhibitors of 5-hydroxytryptophan decarboxylase. It was found that meta-O-methyldopa was a better competitive inhibitor than dopa itself. JB 516 was also active.

A completely new approach to the prolongation of the pharmacological activity of catecholamines was made by the study of compounds competing with catechol-O-methyltransferase. It was first established that methyl-deficient animals showed no difference in their response to norepinephrine with regard to normal control animals. No activity was shown by JB 516, cysteine, methionine and ethionine. However, striking effects were displayed by glycocyamine, nicotinamide, arterenone and adrenolone. All these compounds acted as competitive methyl acceptors. The fate of norepinephrine was followed in vivo in a study involving over 300 mice by determining accurately the levels of 1) norepinephrine, 2) normetanephrine, 3) dihydroxymandelic acid, all in the presence or absence of inhibitors of O-methyltransferase as well as monamine oxidase. These studies for the first time yielded accurate physiological half-life times of norepinephrine, namely 20-25 minutes normally, and 40-70 minutes in the presence of inhibitors.

Significance to bio-medical research and the program of the Institute: The field of monamine oxidase inhibitors has acquired much importance. The only exact methods for assaying MAO inhibitors are being used in the laboratory of Dr. Udenfriend. In order to support these important investigations Dr. Ozaki of this laboratory has been delegated to participate in a program of developing and screening inhibitors in this area. Many pharmaceutical companies supply compounds for this program. They also adopt these enzymatic screening methods in their research program.

Proposed Course of Project: Attempts will be made to develop harmala alkaloids which will be more readily absorbed from the gastro intestinal tract. Reversible inhibitors of MAO and various decarboxylases will be looked for. The program of clinical cooperation will be expanded.

Part B included: Yes

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Udenfriend, S., Creveling, C. R., (NHI), Ozaki, M., Daly, J.W. and Witkop, B. (NIAMD): Inhibitors of Norepinephrine Metabolism in vivo. Arch. Biochem. Biophys. 84, 249-251, 1959.

Serial No. NIAMD- 39

1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Assay of Monoamine Oxidase

Principal Investigator (at NIAMD): Bernhard Witkop

Other Investigators: John Daly

Cooperating Units: Herbert Weissbach, Thomas E. Smith and J. R. Grout, NHI, Serial No. NHI- 208

Man Years (calendar year 1959):
Total: 1/3
Professional: 1/3
Other: 0

Project Description:

Objectives: Major progress was made in the purification of monoamine oxidase when Weissbach succeeded in obtaining preparations devoid of particulate matter. Such soluble enzyme preparations have been enriched 10-20 fold. This and similar work necessitated a reliable and rapid method for the quick assay of monoamine oxidase.

Methods Employed: The underlying idea for the selection of a suitable substrate was the introduction of a reactive ortho substituent such as a primary amino group into a suitably substituted primary amine in which the initially formed imine or aldehyde would undergo self-condensation to a stable product.

Major Findings: All these requirements were met in kynuramine, for which a new synthesis was developed in the oxonolysis of N-carbobenzoxytryptamine. Kynuramine was found by Dr. Weissbach to be a good substrate for monamine oxidase. Its enzymatic disappearance can be followed spectrometrically by the disappearance of the absorption peak at 360 mμ. The product formed in this reaction is 4-hydroxyquinoline, which no longer absorbs at 360 mμ, but at 329 and 315 mμ. Thus, by measuring the decrease in absorption at 360 mμ one has a simple and rapid assay for monamine oxidase.

Significance to bio-medical research and the program of the Institute: This new rapid assay should prove useful in the expanding program on the purification of monoamine oxidase and similar enzymes and on the screening of compounds affecting them.

Proposed Course of Project: Further purification of the enzyme is being contemplated.

Part B included: No

Serial No. NIAMD- 40
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Histochemical Studies on Monoamine Oxidase

Principal Investigator (at NIAMD): Bernhard Witkop

Other Investigators: Dr. Yuichi Kenaoka (V.S., arrived Aug. 31, 1959)

Cooperating Units: Herbert Weissbach and Betty Redfield, NHI, Serial No. NHI- 308 and Dr. G. Glenner, NIH, NIAMD

Man Years:
Total: 1/3
Professional: 1/3
Other: 0

Project Description:

Project: Synthesis of substrates of monoamine oxidase and of D-amino acid oxidase which in vivo might be converted to histochemical stains.

Objectives: Earlier studies by Glenner, Weissbach and Redfield have shown that during the oxidation of tryptamine by monoamine oxidase a concurrent reduction of added diiodonitrotetrazolium chloride took place, the aldehyde from the amine acting as the reducing agent. It has now been attempted to have the features of a histochemical stain built into a possible substrate, such as a primary amine or an α-amino acid for monoamine oxidase or D-amino acid oxidase.

Methods Employed: Derivatives of o-ω-aminoacetophenone and phenylglycine with amino groups in the ortho position of the phenyl ring have been synthesized or are in the process of synthesis. Spectrophotometric assay in situ has been used to follow the disappearance of the substrate or the appearance of oxidation or condensation products, such as indigo. Investigation will proceed to in vivo systems, and histochemical staining phenomena are being looked for.

Major Findings: So far only o-ω-diaminoacetophenone has been subjected to the action of monoamine oxidase and found to be a much poorer substrate than the homologous kynuramine, in the same way that adrenalone is more slowly oxidized by MAO than noradrenaline. The preparation of dihydro-o-ω-diaminoacetophenone is under investigation.

Significance to bio-medical research and the program of the Institute: The localization of the enzymes involved in the breakdown of biogenic amines and amino acids in various tissues and organs has been a matter of considerable interest to histochemists. The approach chosen in this project develops the stain from enzymatic transformation of the substrate rather than from an interaction with an extraneous compound. Such an approach is much more direct and should, if successful, lead to topographic maps of important catabolic enzymes.

Proposed Course of Project: Limitations are imposed upon these synthetic substrates by the specificity of the enzymes. A systematic study on a larger body of compounds will be required to reconcile minimum useful rates of enzymatic oxidation with optimal staining properties of the condensation products thus produced.

Part B included: No

Serial No. NIAMD-41
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Selective Cleavage of the Hydroxyproline Peptide Bond in Model Peptides and in Collagen

Principal Investigator: Bernhard Witkop

Other Investigators: John Francis, A. V. Robertson (V.S., arrived Aug. 26, 1959)

Cooperating Units: K. A. Piez, NIDR, Serial No._____

Man Years (calendar year 1959):
Total: 1-1/3
Professional: 1-1/3
Other: 0

Project Description:

Objectives: To find a selective chemical method for the cleavage of peptide bonds next to hydroxyamino acids, especially hydroxyproline, with a view of applying such cleavage methods to the structural elucidation of collagen and gelatin.

Methods Employed: It had been observed previously in this laboratory that suitable O-tosyl hydroxy-L-proline derivatives, in the presence of proton acceptors, undergo an internal elimination reaction with the formation of allo-hydroxy-L-proline lactones. The principal of this reaction is now being applied to suitable hydroxyproline peptide derivatives.

Major Findings: Although the carboxylate anion readily displaces a trans-O-tosyl group in the natural hydroxyproline series with concomitant lactonization, no such participation occurs in N-carbobenzyloxy-O-tosyl-hydroxyprolylglycine.

Significance to bio-medical research and the program of the Institute: Collagen, the quantitatively most important protein in mammals, is characterized by its high content of hydroxyproline, an amino acid occurring more or less exclusively therein. A special method for the selective cleavage of the hydroxyproline peptide bonds is highly desirable, since most enzymes fail to cleave this bond. Such a method would make it easier to find, as has been reported, the

areas of high hydroxyproline content in the peptide strands of collagen (Grassmann), and would facilitate the analysis of urinary hydroxyproline peptides observed in some patients with metabolic disturbances (Dr. F. Irreverre).

Proposed Course of Project: The reasons for this lack of reactivity are being investigated. N-Carbobenzyloxy-3,4-dehydroproline, its peptide with glycine and other derivatives will be subjected to N-bromosuccinimide and the amount of lactonization or peptide cleavage determined.

At the same time promising leads on partially selective cleavage of N-(hydroxy)proline peptide bonds by sodamide in liquid ammonia will be followed up.

Part B included: No

Serial No. NIAMD 42
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Oxidative Cleavage of Tyrosyl-Peptide Bonds: a specific chemical peptidase

Principal Investigator: Louis A. Cohen

Other Investigators: G. L. Schmir, J. G. Wilson (U.S., arrived Nov. 12, 1959)

Cooperating Units: None

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

Project Description:

A study of the chemical fragmentation of polypeptides and proteins at tyrosyl-peptide bonds by use of oxidizing agents such as bromine and N-bromosuccinimide.

Objectives: To effect the splitting of complex peptides by the addition of a chemical reagent which attacks tyrosine exclusively and labilizes the adjacent peptide bond. To study the use of phloretic acid as a unique and highly specific amine blocking group in peptide synthesis.

Methods Employed: Rapid recording ultraviolet spectroscopy is used to follow the course and extent of cleavage reactions. Infrared spectroscopy is used to elucidate the structure of reaction products. Paper and column chromatography and high-voltage electrophoresis are used to separate and purify polypeptide fragments.

Major Findings: Numerous peptides of tyrosine have been cleaved selectively at the adjacent peptide bond involving the carbonyl group of tyrosine. The octapeptide hormone hypertensin has been cleaved exclusively at its tyrosyl-valine bond.

Significance to bio-medical research and the program of the Institute: The ability to split complex peptides (enzymes and proteins) at specific bonds can contribute greatly to the determination

of amino acid sequences, to the modification of proteins without denaturation and to the isolation of active fragments of enzymes. By these techniques proteins can be split under very mild conditions (neutral pH, aqueous solution) by a rapid controllable reaction at selected positions.

Proposed Course of Project: To extend the study of the selective cleavage of the tyrosyl peptide bond to complex systems such as polypeptide hormones and proteins.

Part B Included: No

Serial No. NIAMD- 43
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Studies on a Bound Form of the Neurotropic γ-Aminobutyric Acid in Brain

Principal Investigator (in NIAMD): Louis A. Cohen

Other Investigators: William M. Jones

Cooperating Units: Jean D. Wilson and John J. Pisano, NHI, Serial No. NHI- 220

Man Years (calendar year 1959):
Total: 1-1/3
Professional: 1/3
Other: 1

Project Description:

In the course of isolation of Coenzyme A from brain a fraction was found by J. J. Pisano which, upon hydrolysis, yielded γ-aminobutyric acid.

Objectives: This occurrence of a bound form of γ-aminobutyric acid in brain raised the question of its chemical structure.

Methods Employed: Purification on charcoal and Dowex-50 columns followed by paper electrophoresis served as methods for concentration and isolation of the bound form of γ-aminobutyric acid whose concentration varies from 500-9,000 µg. per kilogram brain of dog, pig and beef.

Major Findings: The new compound is a peptide hydrolyzable to histidine and γ-aminobutyric acid. γ-Aminobutyryl-L-histidine, obtained by synthesis, had all the properties of the naturally occurring compound.

Significance to bio-medical research and the program of the Institute: The literature on the significance of the neurotropic γ-aminobutyric acid in brain has been growing rapidly during recent years. The occurrence of this amino acid in a bound peptide form with histidine as a homolog of carnosine raises many interesting questions

such as penetration problems, mode of biosynthesis, active and inactive transport forms, etc.

Proposed Course of Project: It will be tempting to synthesize further analogs and homologs of this kind and to subject them to pharmacological and neurological studies.

Part B included: No

Serial No. NIAMD-AA
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Selective Oxidation of Free and Bound Histidine and Other Imidazole Derivatives

Principal Investigator: Louis A. Cohen

Other Investigators: G. L. Schmir

Cooperating Units: None

Man Years (calendar year 1959):
Total: 1
Professional: 1
Other: 0

Project Description:

A study of the oxidative degradation of histidine derivatives by use of reagents such as N-bromosuccinimide.

Objectives: To achieve the oxidative removal of imidazole rings in histidine peptides with a view of possibly effecting the cleavage of peptide bonds adjacent to histidine by an intramolecular reaction involving the disintegrating imidazole ring of histidine; to study the use of arylsulfonyl groups as protecting groups for the imidazole ring in protein degradation and in peptide synthesis; to correlate metal binding and histidine destruction in proteins.

Methods Employed: Recording ultraviolet spectroscopy to follow reactions of sulfonylated imidazoles. Infrared spectroscopy to determine the structures of reaction products. Paper chromatography and paper electrophoresis to help in structure elucidation and purification of histidine derivatives.

Major Findings: The imidazole ring has been rapidly oxidized by N-bromosuccinimide under mild conditions to yield a keto aldehyde, ammonia and formic acid. Ring-nitrogen substituents such as p-toluenesulfonyl protect the ring against oxidative degradation and provide a new route to the synthesis of histidine peptides.

Significance to bio-medical research and the program of the Institute: The general objectives of specific chemical cleavages of peptide bonds lie in the area of protein sequence studies, modifica-

[illegible] There [illegible] achieved by the [illegible] cleavage of tyrosyl [illegible] peptide bonds, [illegible] further if other [illegible] such as those [illegible] histidine, can be also selectively [illegible]

Proposed Course of Research: The study of the action of [illegible] oxidizing agents on histidine peptides and to investigate [illegible] the tosyl group in specific modification of the histidine [illegible] in proteins.

Part B [illegible]

Serial No. NIAMD-45
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Mechanism and Manipulation of the Biosynthesis of Hydroxyproline and of Collagen

Principal Investigator (at NIAMD): Bernhard Witkop

Other Investigators: A. V. Robertson (V.S., arrived Aug. 26, 1959)

Cooperating Units: K. Gibson, S. Udenfriend, NHI, Serial No. NHI-217
A. Berger (V.S. in Dr. Anfinsen's Laboratory)

Man Years (calendar year 1959):
Total: 1-1/3
Professional: 1/3
Other: 1

Project Description:

Objectives: At the outset of this expansive investigation the following limited objectives will be pursued: 1) The synthesis and resolution of 3,4-dehydroproline. 2) The reductive tritiation of dehydroproline to 3,4-^{3}H$_2$-L-proline. 3) The synthesis of other specific (diastereo)isomers of 3-, 4-, and 3,4-tritiated prolines and hydroxyprolines. 4) Synthesis of 3,4-epoxy-L-proline as a key intermediate for further functional derivatives of proline having 2 OH groups, fluoro groups, etc. 5) Synthesis of polydehydro-L-proline as a further model for the existence or nonexistence of two rotational isomers analogous to polyproline.

Methods Employed: Special reduction of pyrrole-2-carboxamide following E. Fischer's method yielded 3,4-dehydro-DL-proline and its amide whose structures were proven by reduction to proline and proamide and by NMR spectroscopy. Resolution of the amide was achieved by chemical and enzymatic methods. Tritiation is in progress.

Major Findings: The new amino acid in two-dimensional paper chromatograms is very close to proline, gives a yellow ninhydrin spot but is not identical with any unknown spots of this color from marine or animal collagen. The rotatory contribution of the new double bond is high and makes for a total $(\alpha)_D = -209°$.

Significance to bio-medical research and the program of the Institute: K. Gibson in the laboratory of S. Udenfriend will continue

the biosynthetic studies on collagen started by Ch. Mitoma. The mechanism of hydroxylation of proline (free or bound) will be studied with as many proline derivatives tritiated in selected positions as possible. The competitive inhibition of dehydroproline, 4-fluoro-proline etc. will be studied.

Part B included: No

Serial No. NIAMD- 46
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Studies with the Enzyme, O-Methyltransferase

Principal Investigator (at NIAMD): Bernhard Witkop

Other Investigators: John Daly, Siro Senoh (left 3/17/59)

Cooperating Units: S. Udenfriend and associates, NHI, Serial No. NHI- 216
J. Axelrod, NIMH, Serial No. M-CS-PM-3

Man Years (calendar year 1959):
Total: 2/3
Professional: 2/3
Other: 0

Project Description:

Objectives: As shown by Axelrod and coworkers, the enzyme O-methyltransferase is primarily responsible for the metabolism of epinephrine and norepinephrine. Because of the great interest in catechols and in their metabolism a thorough study of the action of O-methyltransferase on a variety of substrates has been initiated.

Methods Employed: The enzymatic O-methylation of various catechols has been studied in vivo and in vitro, and the products investigated. Periodate oxidation of meta- and paranephrine derivatives has led to vanilline and isovanilline whose separation is rapid and quantitative.

Major Findings: In vitro studies using O-methyltransferase and various catechols as substrates has led to the interesting finding that not only does O-methylation occur meta to the side chain as reported in the literature, but that a significant amount of para-O-methylation also occurs. The amount of this para-O-methylation varies according to the nature of the side chain. For compounds containing electron-withdrawing groups in their side chains such as acetovanillone, arterenone and adrenalone, the para isomer totals 40-56% of the methylation product, while with compounds containing saturated side chains such as 3,4-dihydroxyphenylmethylcarbinol, dopamine, epinephrine and norepinephrine, the para isomer is formed only to the extent of 10-15%. The occurrence of p-O-methylation in vivo is of great interest, and with acetovanillone, arterenone, and adrenalone, p-O-methylation has been demonstrated in the intact rat

although to a lesser extent than in vitro. A possible explanation of this was found for acetovanillone when it was shown that the para and meta O-methylated derivatives of acetovanillone undergo a novel interconversion in vivo, with the para compound being most labile to conversion. Studies were undertaken to demonstrate the formation of para-O-methylated epinephrine (paranephrine) in vivo but the results, in contrast to the in vitro studies, indicate no formation of paranephrine.

Studies on the half-life time of norepinephrine administered to mice showed no effect with monoamine oxidase inhibitors while various O-methyltransferase inhibitors almost doubled the half-life time of norepinephrine, an important pharmacological finding.

3,4,5-Trihydroxyphenethylamine, shown to be an inhibitor of O-methyltransferase in these studies, is also of interest as tridesmethyl mescaline. Studies have been initiated on the methylation of this compound and also on the enzymatic demethylation of mescaline, and the products are being investigated.

Significance to bio-medical research and the program of the Institute: The great importance of catecholamines in regard to central and peripheral neurochemistry lends great interest to the function of enzymes such as O-methyltransferase which effect their metabolism.

Proposed Course of Project: The biochemistry and metabolism of mescaline, and tridesmethylmescaline in reference to the enzymes O-methyltransferase and O-demethylase will be investigated further. The possible formation of Coenzyme Q, recently reported as an important oxidation-reduction coenzyme, from a tetrahydroxybenzene derivative through the action of O-methyltransferase, will be investigated.

Part B included: Yes

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Senoh, S., Daly, J.W., Axelrod, J. and Witkop, B.: Enzymatic p-O-Methylation by Catechol O-Methyl Transferase. J. Am. Chem. Soc., 81, 6240-6245, 1959.

Serial No. NIAMD- 47
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Regulation of Growth of Animal and Plant Cells by Derivatives of Natural Hydroxyamino Acids

Principal Investigator: Bernhard Witkop

Other Investigators: A. V. Robertson (V.S., arrived Aug. 26, 1959)

Cooperating Units: M. Gibson, NIH, Serial No. NHI- 217
Dr. F. C. Steward, Cornell University
Dr. S. Archer, Sterling-Winthrop Research Institute

Man Years (calendar year 1959):
Total: 2/3
Professional: 2/3
Other: 0

Project Description:

Project: To determine the influence of hydroxyamino acids and their analogs as possible regulators or inhibitors of cellular growth.

Objectives: To accomplish a more direct control of protein synthesis, tissue regeneration, formation of collagenous scar tissue by direct and local application of cyto-active agents than was hitherto possible by remote and hormonal control.

Methods Employed: A plant tissue culture system has been used to detect the growth inhibitory effect of a number of nitrogenous compounds and to determine, where possible, the metabolic site at which the substance in question may act. The tissue culture system consisted of explants from carrot root stimulated to grow by cell division. This was suggested because the cells which grow in this way synthesize a protein in which proline is incorporated and which is unusually rich in hydroxyproline for a plant protein.

At the moment the actinomycin producing strain of streptomyces is the only microorganism known to incorporate unusual and foreign amino acids such as hetoproline, pipecolic acid, azetidinecarboxylic acid into the peptide part of the antibiotic which it elaborates. Dr. Katz is the only expert known or available at the present time who masters the technique of following the incorporation of these foreign

amino acids and of the analysis of the resulting modified actinomycin.

Major Findings: 1) In plant tissue: see publication by Steward, Pollard, Patchett and Witkop: "The Effects of Selected Nitrogen Compounds on the Growth of Plant Tissue Cultures," Biochimica et Biophysica Acta, 28: 308, 1958. 2) In chicken embryos ketoproline, an analog of hydroxyproline, causes a prolonged elevation of free hydroxyproline. The mechanism for this elevation has been established to be due to inhibition of hydroxyproline catabolism by ketoproline and by enzymatic conversion of ketoproline to hydroxyproline. The enzyme for the latter reaction is found in the supernatant fraction of rat kidney and liver, and requires reduced pyridine nucleotide. Some of these findings have been summarized at the 4th International Congress of Biochemistry, Abstracts, Section 12, No. 27, p. 152.

Significance to bio-medical research and the program of the Institute: The existence of enzymes capable of reducing ketoproline to hydroxyproline raises the question of a reverse transformation which, on the level of collagen, could lead to the formation of cross-linkages. Whether the hardening of collagen with age may be due to such a sequence of reactions remains to be seen.

Proposed Course of Project: Drs. Gibson and Udenfriend will study the effect of prolonged administration of ketoproline on animals and will try to purify the enzyme involved in the conversion of ketoproline to hydroxyproline. The synthesis of 3-hydroxy-, 3,4-dihydroxy, 3- or 4-fluoroprolines will be attempted.

Part B included: No

1. Chemistry
2. Metabolites
3. Bethesda.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The Chemistry and Metabolic Fate of Tryptamine

Principal Investigator (at NIAMD): Bernhard Witkop

Other Investigators: John Daly

Cooperating Units: S. Udenfriend and H. Weissbach, NHI, Serial No. [illegible]
Regis Chemical Co., Chicago (custom synthesis of certain hydroxyindoles)

Man Years (calendar year 1959):
Total: 1-2/3
Professional: 2/3
Other: 1

Project Description:

Objectives: The discovery of Dr. Udenfriend that tryptamine arises from tryptophan by the action of a new decarboxylase, and that it occurs in significant amounts in the brain, focuses attention on this new biogenic amine and its transformations.

Methods Employed: Non-enzymatic and enzymatic oxidations of cold and radioactive tryptamine lead to a number of new compounds which are under investigation. Nuclear magnetic resonance spectrophotometry is utilized to gain information on the possible existence of unstable tricyclic tautomers of tryptamine.

Major Findings: The appearance of tryptamine in the brain has raised the question of its possible transformation to serotonin. Such a conversion, however, has now been ruled out, since radioactive tryptamine in vivo does not lead to radioactive serotonin. The nuclear magnetic resonance spectra of tryptamine derivatives leave no room for the assumption of a small amount of a labile tricyclic tautomer. Oxidation of tryptamine by a microsomal enzyme system has been shown to lead to a new hydroxytryptamine not identical with serotonin. In analogy to some Japanese findings this new metabolite has been considered as 7-hydroxytryptamine. However, recent results have proved the structure of a 6-hydroxytryptamine. Through Sandoz Pharmaceuticals, Basle, Switzerland and the Regis Chemical Co., a number of 6- and 7-hydroxyindoles have become accessible.

Significance to bio-medical research and the program of the Institute: The recent finding that the Mexican mushrooms used for producing central effects in religious rituals contain as their active ingredient psylocybin, i.e., the O-phosphate of 4-hydroxy-N,N-dimethyltryptamine, imparts special interest to the novel hydroxytryptamine metabolites mentioned above.

Proposed Course of Project: The chemistry and psychopharmacology of 6- and 7-hydroxyindoleethylamines will be investigated.

Part B included: Yes

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Udenfriend, S., Creveling, C. R., Posner, H., Redfield, B. G., Daly, J, and Witkop, B.: On the Inability of Tryptamine to Serve as a Precursor of Serotonin. Arch. Biochem. Biophys. 83: 501-507, 1959.

Serial No. NIAMD- 40

1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Higher-Carbon Sugars, Anhydro Sugars, Amino Sugars, Sugar Alcohols and their Derivatives.

Principal Investigator: Nelson K. Richtmyer

Other Investigators: Hans Helmut Baer (V.S. from 9/8/59), Alexander J. Charlson (V.S. until 11/13/59), James W. Pratt (until 4/20/59), Hugo H. Sephton (V.S. from 9/1/59), Emmanuel Zissis, John F. Sipos, Edward W. Tracy

Cooperating Units: None

Man Years:
Total: 5
Professional: 3 2/3
Other: 2 1/3

Project Description:

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

Methods Employed and Major Findings: In continuation of our examination of the higher-carbon sugars in the avocado and Sedum species we have shown that the same octulose occurs in both of these plant materials and have established its structure as D-glycero-D-manno-octulose by degradation and by cyanohydrin synthesis from D-glycero-D-manno-heptose. We have isolated from the avocado the first known naturally occurring octitol and have proved its structure to be D-erythro-D-galacto-octitol; we have found evidence also for the probable presence of D-talo-heptulose in the avocado. We have isolated β-sedoheptitol (D-glycero-D-gluco-heptitol) from Sedum; this is the first reported occurrence of this sugar alcohol in nature.

Studies on the formation of anomeric nonreducing sugars were continued, with a considerable amount of time being devoted to the preparation of starting materials and intermediates; studies on amino sugars derived from higher-carbon sugars were begun.

Proposed Course of Project: Continuation of these and closely related topics.

Part B included: Yes

Part 3. Honors, Awards, and Publications

Publications other than abstracts from this project:

Charlson, A. J. and Richtmyer, N. K.: Isolation of D-glycero-D-manno-Octulose from the Avocado. J. Am. Chem. Soc., 81: 1512, 1959.

Serial No. NIAMD- 50

1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

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

Principal Investigator: Hewitt G. Fletcher, Jr.

Other Investigators: R. Barker (V.S. from 8/28/59), E. W. Diehl, D. L. MacDonald, R. K. Ness, C. Pedersen (Fellow), H. B. Wood, Jr.

Cooperating Units: None

Man years (calendar year 1959):
Total: 7 1/3
Professional: 5 2/3
Other: 1 2/3

Project Description:

General Objectives:

A. To investigate new synthetic pathways for the synthesis of carbohydrate substances of importance to medical research.

B. To make difficultly accessible carbohydrate derivatives available to medical researchers either through direct gift or through publication of directions for the preparation thereof.

C. To extend knowledge of the chemical properties of biochemically important carbohydrates.

Specific Objectives: To study the chemistry of D-ribose, 2-deoxy-D-ribose and other sugars with the objective of synthesizing substances which have been demonstrated to be (or suspected of being) intermediates in carbohydrate metabolism.

Progress during 1959:

A. An improved synthesis of 2-deoxy-D-ribose, suitable for relatively large-scale production of this sugar has been evolved.

B. A method has been devised for the synthesis of 2-deoxynucleosides.

C. The benzoylated D-ribosyl and L-arabinosyl fluorides have been investigated with three noteworthy results: (a) a transformation from the D-ribopyranose to the D-ribofuranose series, (b) a facile conversion from the arabinose to the ribose series and (c) a ready route to ribofuranose and ribopyranose derivatives substituted at carbon two.

D. 2-Deoxy-D-ribofuranose 1-phosphate has been synthesized by chemical means for the first time.

E. The reaction between various 1-thioaldose derivatives and certain heavy metal salts has been shown to provide a new synthetic pathway to 1-substituted aldose derivatives.

F. The preparation of 3-deoxy-D-glucose and 3-deoxy-D-mannose from 2-deoxy-D-ribose has been achieved.

Significance of the project to the program of the Institute:

The synthetic methods and the materials produced in the course of this project have been and will be of utility to various research groups in NIAMD.

Proposed Course of Project:

The area described under "Specific Objectives" will be pursued during 1959.

Part B included: Yes

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

MacDonald, D. L. and Fletcher, H. G., Jr.: 2-Deoxy-D-ribose. III. The Synthesis of 2-Deoxy-D-ribose 5-Phosphate. J. Am. Chem. Soc. 81, 3719-3722, 1959.

Ness, R. K. and Fletcher, H. G., Jr.: Synthesis of the Two Anomeric 9-(2-Deoxy-D-Pibofuranosyl)-Adenines. J. Am. Chem. Soc., 81, 4715 1959.

Serial No. NIAMD-EB
1. Chemistry
2. Analgesics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project title: Chemical structure and action of morphine-like analgesics.

Principal Investigator: Nathan B. Eddy.

Other investigators: None

Man Years: Total : 3.33
Professional: .66
Other : 2.66

Project description:

Some thirty odd compounds have been made this year in the Section's laboratory, notably benzomorphans and the new series of aminoacetates and aminobutyrates. In addition well over one hundred compounds have been received from laboratories in the United States and in Europe. In addition to quantitative comparisons of the series of compounds being built up by our own chemists, a large group of compounds related to pethidine and another group related to levorphanol are being evaluated, partly for the accumulation of data and structure-action relationship and partly in an effort at further separation of useful analgesic action, from addiction liability and side action incidence (see last year's report). Our major contribution in this effort, phenazocine, is now ready for market after trials in thousands of patients. The Government has allowed foreign rights in this compound to lapse and its introduction abroad by private industry is anticipated.

The few patients in the Clinical Center which have come to our attention this year on account of their pain problems, have been treated with oral doses of phenazocine. Good relief has been obtained with as little as 2.5mg., when the patient has had little previous narcotic experience. If the patient was tolerant to a previously used opiate a larger dose was required but it is our impression that cross-tolerance is incomplete. No side effects have been encountered with adequate analgesic doses.

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A good start has been made with the Coded Information Center, supported in part by NIMH. Several thousand documents have been coded and keysort cards, author index cards, etc., have been made for well over a thousand of these. We are beginning with current and very recent literature and the accumulation is already proving a very useful source of information for ourselves and others.

As a part of our consultative service to other Government agencies a significant contribution was made to the legislative program for improvement in the national narcotics control regimen. Conferences with the Addiction Research Center of NIMH, the Bureau of Narcotics, the National Research Council, and the sponsor of the legislation, have resulted in amendment of HR-529, to be known as the Narcotic Act of 1959, providing for flexibility in narcotics control, modification of control in either direction as experience warrants and technical advice to establish a degree of control commensurate with the risk to public health.

Part B included: Yes.

PMS-NIH
Individual Project Report
Calendar Year 1959

Part B:

Publications:

The analgesic equivalence to morphine and relative side action liability of oxymorphone (14-hydroxydihydromorphinone), by Nathan B. Eddy and Lyndon E. Lee, Jr. J. Pharmacol. (1959) 125, 116.

The rate of development of physical dependence and tolerance to analgesic drugs in patients with chronic pain. I. Comparison of morphine, oxymorphone and anileridine, by Nathan B. Eddy, Lyndon E. Lee, Jr., and Carl A. Harris. Bull. Narc. (1959) 11, No. 1, 3.

The rate of development of physical dependence and tolerance to analgesic drugs in patients with chronic pain. I. Comparison of morphine, oxymorphone and anileridine. Condensation in French. Nathan B. Eddy, Lyndon E. Lee, Jr., and Carl A. Harris. Bull. WHO Hlth Org. (1959) 20, 1245.

Structures related to morphine. XII. Synthesis of 2'-hydroxy-5,9-dimethyl-2-phenethyl-6,7-benzomorphan (NIH 7519) by E. L. May and Nathan B. Eddy. J. Org. Chem. 24 (October 1959).

A new potent synthetic analgesic by Nathan B. Eddy with Everette L. May. J. Org. Chem. (1959) 24, 2

Addiction Liability & Narcotics Control by Nathan B. Eddy and Harris Isbell. Public Health Reports (1959) 74, No. 9, 755.

Chemical structure and action of morphine-like analgesics & related substances, by Nathan B. Eddy. Chemistry & Industry (England) (1959) 21 November No. 47, pp. 1462-1469.

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Part B (continued)

Honors and Awards:

Secretary, Committee on Drug Addiction and Narcotics, National Research Council - Reappointment.

Oct. 19-24, participated in 10th Session, Expert Committee on Addiction-producing Drugs, World Health Organization, Geneva, Switzerland.

Oct. 1 - Lister Memorial Lecturer, Edinburgh, Scotland.

Oct. 2 - Member of Panel, Symposium on Analgesics, Edinburgh, Scotland.

Oct. 1 to Oct. 31 visited laboratories working on analgesic problems. - Edinburgh, Scotland; Meerse, Belgium; Louvain, Belgium, Ingelheim, Germany; Basel, Switzerland; Copenhagen, Denmark; Stockholm, Sweden.

Oct. 6 - Addressed postgraduate classes, Louvain University, Louvain, Belgium on "Methods for Determining Addiction Liability."

Oct. 30 - Addressed Drug Control Unit, Karolinska Institute, Stockholm, Sweden on "Addiction liability and narcotics control".

Serial No. NIAMD-32
1. Chemistry
2. Aa
3.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project title: The effect of the administration of thyroxin on the recovery of N-demethylase after abrupt withdrawal of narcotic drugs.

Principal Investigators: Joseph Cochin and Louis Sokoloff (NIMH).

Other investigators: None

Cooperating Unit: NIMH

Man years: Total : 1/3
Professional: 1/3
Other : 0

Project description:

Objectives - To determine whether the administration of thyroxin before and during abrupt withdrawal of morphine affects the resynthesis of the N-demethylase diminished during the period of chronic morphine administration.

Methods and results:

The administration of 90 micrograms of l-thyroxin to rats for periods of 7 days does not affect th N-demethylase, but three days after withdrawal of 7-day hyperthyroid animals that have also been given morphine chronically, N-demethylase activity is about half of that of animals treated with morphine alone. Thus there seems to be a real potentiation of the effect of morphine by thyroxin. Fourteen day treatment with thyroxin alone depresses activity of the enzyme moderately, but the combination of morphine plus thyroxin results in a more profound depression of demethylase activity far greater than that following narcotic drugs alone. The recovery of N-demethylase activity after withdrawal is delayed significantly by making the animals hyperthyroid prior to withdrawal.

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Significance to the program of the Institute:

Contribution to the understanding of the effect of thyroxin on microsomal enzyme systems and a possible lead toward understanding the mode of action of the morphine effect on N-demethylation.

Proposed course of the project:

It is hoped to continue the project in-vivo by varying time intervals of thyroid administration and by using thyroidectomized animals and thyroid blocking drugs. It is also planned to carry out extensive in-vitro experiments to see whether this effect can be reproduced by addition of thyroxin to tolerant non-tolerant liver preparations. Attempts will be made to simplify the in-vitro system in order to localize the site of action and investigate the mechanism of this effect.

Part B included: No.

Serial No. NIMH - 54
1. Chemistry
2. Analgesics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project title: Biochemical and pharmacological changes after chronic administration of narcotic drugs.

Principal Investigator: Joseph Cochin.

Other investigators: Julius Axelrod (NIMH)

Cooperating Unit: NIMH - M-CS-PH-3

Man years: Total : 1/3
Professional: 1/3
Other : 0

Project description:

Objectives: a) To determine whether depression of N-dealkylation of narcotic drug substrates by rat-liver homogenates after chronic administration of narcotic drugs parallels diminution of *in-vivo* responses and whether changes in the enzymes involved can serve as a model for the *in-vivo* changes with tolerance.

b) To investigate the nature of the reduction in enzymatic activity.

Methods and Results:

a) (1) In an attempt to correlate the *in-vitro* N-dealkylation of a series of compounds of the morphine series with their relative analgesic potency *in-vivo*, efforts were made to develop methods of extraction and separation of the enzymatically dealkylated nor compound from the parent N-substituted morphinan in order to determine the role of dealkylation. We have had only partial success in these attempts and a clean separation of closely related morphinans is not yet possible.

2) Continuation of the studies of parallelism of the *in-vitro* and *in-vivo* changes accompanying tolerance revealed that the stereospecificity heretofore considered a function of analgesic potency, that is, that the isomer which was the more potent analgesic was also the one

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which was dealkylated more readily, was true only in a particular species, and that in another species, the reverse might well be true.

b) It was found that the dealkylation reaction as it had been described heretofore was not saturated with respect to TPN and that increasing TPN 10-20 fold and adding glucose 6-PO4 boosts the rate of dealkylation five to eight times. However, the ratio of activity of normal and tolerant livers remains about the same, indicating that the defect in the tolerant animals is probably not one of co-factor deficiency but rather a true destruction or blocking of enzyme activity. Attempts to purify and isolate the N-demethylase have not been successful thus far.

Significance to the program of the Institutes:

We believe this to be a contribution to the understanding of the mechanism of tolerance.

Proposed course of the project:

We hope to continue these studies of the relationship of the rate of N-dealkylation and analgesic efficiency and/or dependence liability. We also propose to continue attempts to purify, to some extent at least, the crude microsomal enzyme we are at present using.

Part B included: Yes.

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Part B:

Publications:

Cochin, J. and Axelrod, J.: Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalorphine and normorphine. J. Pharm. & Exper. Therap., 125: 105, 1959.

Honors and awards:

Gave seminar on "Biochemical and pharmacological changes accompanying tolerance at NIMH Addiction Research Center, Lexington, Ky., January 1959; at Dept. of Pharmacology, George Washington University, May 1959; at Dept. of Pharmacology, Emory University, Atlanta, Georgia, Sept. 1959.

Serial No. NIMH-34
1. Chemistry
2. Analgesics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project title:

1) Chemistry and neuropharmacologic study of compounds derived from 3,4-dihydro-7-methoxy 2(1H)-naphthalenone.

2) Stereochemical direction of addition to the carbonyl group of 2'-methoxy-2,5-dimethyl-9-oxo-6,7-benzomorphan (oxycodone and oxymorphone analogs).

3) Preparation of miscellaneous benzomorphans.

Principal Investigator: Everette L. May

Other Investigators: Hiroshi Kugita & J. Harrison Ager

Cooperating units: Smith, Kline & French Laboratories, Department of Pharmacology, University of Michigan and Addiction Research Center, Lexington, Ky. Clinical complementary to pharmacological investigations in this Section.

Man Years: Total : 2.5
Professional: 2.5
Other : 0.0

Project description:

Objectives: To add to our basic knowledge of [illegible] chemistry and of chemical structure - neuropharmacologic behavior implications; to synthesize [illegible] medicinal agents.

Methods employed: The standard and the more [illegible] techniques of organic chemistry including spectral methods of analysis, as well as pharmacologic evaluation methods.

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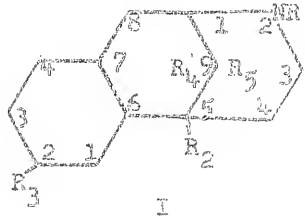
Major findings: 1) A more versatile approach to the pharmacologically and chemically interesting benzomorphan (I) family of compounds developed by our laboratory was sought and found. This approach



I

involves 3,4-dihydro-7-methoxy-2(1H)-naphthalenone as a starting material and 2'-methoxy-2,5-dimethyl-9-oxo-6,7-benzomorphan (II) (I, R_1=R_2=Me, R_3=OMe, R_4R_5=oxo) as an interesting intermediate. In the synthesis of the latter a pyrolysis reaction on the methiodide was necessary. This pyrolysis conducted by dry distillation yielded mainly tar but also a small amount of an α,β-unsaturated ketone resulting from elimination of HI and nitrogen ring opening (Hofmann degradation). The structure of this compound was readily proved by standard methods. The desired compound II was finally obtained in satisfactory yield along with varying amounts of the Hofmann product (depending upon the solvent used) by conducting the pyrolysis in hexanol, heptanol or octanol; octanol was optimal. Surprisingly, in the presence of acids compound II formed very stable hydrates (or alcoholates) at the carbonyl group as shown by analysis and infra red study of several of its salts. Regeneration of the free carbonyl group as shown by analysis and infra red study of several of its salts. Regeneration of the free carbonyl group with base was instantaneous. Finally, II was converted in 8 steps to 2'-hydroxy-5-methyl-9-phenethyl-6,7-benzomorphan (R_1=CH_2CH_2Ph, R_2=CH_3, R_3=OH,

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R_1=R_2=H) the 9-demethyl homolog of the clinically promising phenazocine. This 9-demethyl compound, although only about half as potent as phenazocine in analgesic activity, was at least 4 times as active as morphine in the mouse.

2) Addition of H to II or CH_3-H by means of organometallic reagents (CH_3Li, CH_3MgI) was found to be stereochemically controllable. With II as the methiodide (positively charged N) one diastereoisomeric 9-carbinol (with apparently the hydroxyl cis to the iminoethano system, equatorial for the hydro aromatic ring as indicated by spectral data and degradative experiments) is formed to the exclusion of the other. This addition can be almost completely reversed to give the opposite configuration at C_9 if one starts with the free base (negatively charged N) of II. The resulting carbinols (III) (I, R_1=CH_3, R_2=CH_3, R_3=OH or OCH_3, R_4=OH, R_5=N or CH_3) may be looked upon as analogs of oxycodone and oxymorphone (the diastereoisomeric forms of which are unknown) clinically useful drugs of the morphine series, and are being evaluated pharmacologically. Some show interesting properties.

3) The synthesis, optical resolution, and evaluation of benzomorphans more closely related to phenazocine (NIH 7519) have continued. While analgesic activity is practically nil in the N-ethyl, propyl and butyl derivatives corresponding to phenazocine, the N-amyl homolog is equivalent to morphine and shows low physical dependence capacity in the monkey. Furthermore, it has been possible by optical resolution to effect a separation of neuropharmacological action and adverse side actions. For example, levorotatory 2'-hydroxy-2,6,9-trimethyl-6,7-benzomorphan (I, R_1=R_2=R_3=CH_3, R_4=OH, R_5=H) comparable to morphine is twice as potent as the racemate, has a much lower acute toxicity and is rated very low in physical dependence capacity in the monkey as compared with low for the racemate. Finally, levo-2'-methoxy-5,9-dimethyl-2-phenethyl-6,7-benzomorphan (I, R_1=CH_2CH_2

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R_2=R_5=CH_3), (R_3=OCH_3, R_4=H) is as potent in mice as morphine with no physical dependence capacity in the monkey.

Significance of the program to the Institute:

Research in the field of neuropharmacologic agents has pertinence in the area of pain and anxiety states associated with Arthritis & Metabolic diseases.

Proposed course of project:

Present plans are to continue along lines suggested by the major findings above.

Part B included: Yes.

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Calendar Year 1959

Part B:

Publications:

E. L. May and N. B. Eddy: A new potent synthetic analgesic. J. Org. Chem., 24, 294 (1959).

E. M. Fry and E. L. May: Mannich derivatives of analgesic agents. J. Org. Chem., 24, 116 (1959).

E. L. May and J. H. Ager: Structures related to morphine. XI. Some analogs and a diastereoisomer of 2'-hydroxy-2,9,9-trimethyl-6,7-benzomorphan. J. Org. Chem., 24 (October 1959).

E. L. May and N. B. Eddy: Structures related to morphine. XII. Synthesis of 2'-hydroxy-5,9-dimethyl-2-phenethyl-6,7-benzomorphan (NIH 7519). J. Org. Chem., 24 (October 1959).

E. L. May: Chapter on Analgesics in Burger's Medicinal Chemistry, 2nd Ed. (in press) (Interscience).

Honors and awards:

Received "Alumnus of Year" award from Bridgewater College, Virginia, in May 1959.

Presented lecture "Synthetic analgesics" to Hoffmann-La Roche Laboratories in Nutley, New Jersey in February 1959.

Presented lecture on "Analgesics" to members of the Philadelphia Section of the A.C.S., as part of a series of 10 lectures in Medicinal Chemistry in April 1959.

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1. Chemistry
2. Analgesics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project title: Synthetic Analgesics

Principal Investigator: James G. Murphy.

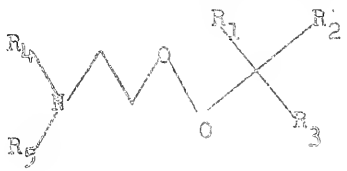
Other investigators: None.

Cooperating Units: None

Man Years: Total : 1.0
Professional: 1.0
Other : 0.0

Project description:

Analogs of Acetylcholine. - Because of the possible role of acetylcholine in sensory nerve transmission, a group of analogs (I) has been prepared in which by



I

progressive substitution of methyl for hydrogen in groups R_1, R_2 and R_3 a graded steric hindrance at the ester linkage is produced with the view of attaining a competitive inhibitor for acetylcholinesterase. Also prepared, have been analogs in which one or both N-methyl (R_4, R_5) has been replaced by phenethyl, a substituent which has been shown to produce augmentation of pharmacological response not only in analgesics but also in other classes of medicinals.

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Thus far no response has been shown for the members which are disubstituted with methyl groups on nitrogen (R_4=R_5=CH_3-), nor for members bearing a single phenethyl group (R_4=CH_3-; R_5=  -CH_2CH_2-), but definite neurotropic activity (analgesic action) has appeared when the nitrogen is doubly substituted by the phenethyl group.

As a byproduct of this work seven substances have been released for cancer screening.

Part B included: Yes

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Part B:

Publications: Synthesis of aminohydrophenanthrene analogs of morphine. James G. Murphy, J. Org. Chem. In press.

Honors & Awards:

PhD. - Georgetown University, June 1959.

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1. Chemistry
2. Analgesics
3. Bethesda

PRS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project title: Mr. Perrine has been on loan to Rocky Mountain Laboratory, Hamilton, Montana for two years. A brief summary of the work done there follows:

Principle Investigator: Theodore D. Perrine

Other Investigators: None

Cooperating Unit: NIAID

Man Years: Total : 1.0
Professional: 1.0
Other : 0.0

Project description:

Enteritides Endotoxin studies. Endotoxin preparations were treated with some 90 organic liquids, and the mixtures then examined for gross solvent action; and subsequent to evaporation of the liquid, for effect on the endotoxin and (in some cases) antigenic potency. About 10 good solvents were found, and the rule formulated that strongly basic amines, and reagents which liberate strong mineral acids or formic acid on mild hydrolysis, will have a deleterious effect on the endotoxin. Reports on the effect on antigenicity are not yet available.

TB Cell Walls. A production model press was designed and constructed, which appears to be applicable to the preparation of all types of bacterial cell walls. This has been written up and should be published shortly.

Vi Antigen. Cooperation on this project with Dr. Jarvis was more or less terminated by Executive Order. However, two publications will probably result.

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Synthetic antigen. Work was mainly concerned with the preparation of vinyl glucoside. This substance has, so far, eluded us but we think we are well on the way to a successful synthesis.

Considerable experience was gained in the techniques of preparing synthetic high-polymers.

Currently returned to the Section on Analgesics and resuming work on the syntheses in the pethidine series, more particularly in the preparation of quinuclidine derivatives.

Part B included: yes.

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Part B:

Publications: The work at Rocky Mountain Laboratory has resulted in the following publications and is expected to yield five additional publications:

Magnetically stirred separatory funnel, by E. Gerwin and T. D. Perrine. Sent to Angew. Chem. 7/20/59

Endotoxic and antigenic fractions from the cell wall of S. Enteritidis methods for separation and some biologic activities, by E. Ribi, K. C. Milner, and T. D. Perrine. J. Immunol., 82, 75 (1959).

Use of a pressure cell for the preparation of cell-free extracts of mycobacteria, by E. Ribi, T. D. Perrine, R. Haas, B. Brown and J. Goode. Proc. Soc. Exptl. Biol. Med., 100, 647 (1959).

Physical and chemical analysis of endotoxin from S. enteritidis, by E. Ribi, B. Meyer, K. C. Milner, T. D. Perrine and C. Larson. J. Immunol. In press.

Method for attaching glass water aspirators to water lines. Unpublished. by T. D. Perrine. Accepted by J. Chem. Ed., Oct. 1959.

Honors and awards: None.

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1. Chemistry
2. Analgesics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project title: (a) The chemical transformation of certain opium alkaloids (or their derivatives) into novel, medicinally useful drugs.

(b) The structure of the so-called "Hydroxycodeine".

Principal Investigator: Lewis J. Sargent

Other Investigators: None

Cooperating Unit: V. Weiss, LPB, NIAMD, Serial No. 119

Man years: Total : 1
Professional: 1
Other : 0

Project description:

(a-1) To determine the effect on analgesic activity of altering the point of attachment of the nitrogen ring from carbon-9 to carbon-7 in the morphine system (using dihydrocodeinone).

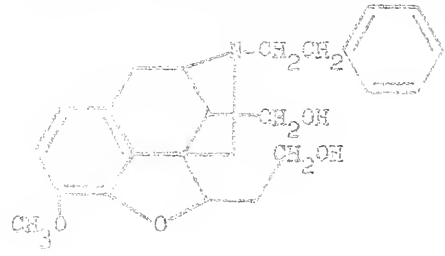
(a-2) In view of the highly encouraging pharmacological results obtained with the recently synthesized NIH 7519, an attempt was made to convert Δ-7,8-desoxycodeine into an analog of the above in which the 4,5-oxygen bridge remained intact. This should afford pertinent pharmacological data relevant to the importance of the dihydrofuran cycle in such systems.

(b) Clarification of the presumed bimolecular structure of "Hydroxycodeine".

Methods employed:

(a-1) The initial sequence of reactions leading to this new class of morphine derivatives, in which the nitrogen terminus of the ethanamine ring was shifted from carbon-9 to carbon-7, was described in the preceding report. Infrared analyses have, in the interim, necessitated altering our conception of the structure of the new isomer. The absence of hydroxyl absorption led to the conclusion that no 4,5-oxygen fission occurred during the decomposition of the quaternary methobromide, and that the product must in fact be a new isomer of dihydrocodeinone. This is being investigated.

(a-2) In pursuing the idea of transforming a derivative of a naturally occurring opium alkaloid to an analog of the potent synthetic analgesic NIH 7519, an unfortunate impasse turned up in the attempted lead tetraacetate cleavage of 7-hydroxycodeine (6,7-glycol). The intermediate aminodialdehyde proved to be exceedingly sensitive and polymerized to intractable products before it could be reduced with lithium aluminum hydride. This difficulty was eventually circumvented by operating on the neutral N-phenacyl derivative of the glycol. However, because of the greater accessibility of the corresponding 7,8-glycol this new approach was pursued as follows: Δ-7,8-desoxycodeine was converted to the nor-cyano derivative and hydroxylated with osmium tetroxide. Acid hydrolysis afforded the nor-glycol which was selectively N-phenacylated and then cleaved with lead tetraacetate. The intermediate N-acyl dialdehyde (which appeared now to be stable) was reduced with lithium aluminum hydride whereupon the following, crystalline N-phenethyl dicarbinol was presumably formed:



(b) Further evidence in support of the bimolecular nature of "hydroxycodeine" was obtained through preparation of the hitherto unknown monoxime and intermediate dihydro derivative, thus completing this project.

Major findings:

The successful transformation of two opium alkaloid derivatives into the novel ring systems described under (a-1) and (a-2) should lead to clinically promising analgesics.

Significance to the program of the Institute:

The search for new drugs (whether synthetic or chemically-modified naturally occurring ones) capable of controlling severe clinical pain, with a minimum of undesirable side-effects, properly falls within the purview of the Institute program.

Proposed course of project:

(a-1) Attempts will be made, in this area, to hydrolyze the 3-methoxyl function as well as to replace N-methyl by the N-phenethyl group.

(a-2) Abolition of the two alcoholic groups (via lithium aluminum hydride reduction of the di-tosyl derivatives) followed by hydrolysis of the 3-methoxyl function should, it is hoped, lead to the desired compound.

Part B: Yes

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Part B:

Publications:

Non-symmetrical Bimolecular Reduction: Structure of the so-called "Hydroxycodeine", by Lewis J. Sargent and Ulrich Weiss. J. Org. Chem., In Press.

NIAMD No. 58
1. Analgesics
2. Chemistry
PHS-NIH 3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project title: Synthesis and use of dihydropyridine derivatives.

Principal Investigator: Edward M. Fry

Other investigators: None

Man years: Total : 1.0
Professional: 1.0
Total 0.0

Project description:

Objectives: To develop a method of alkylating the 2 and 3 positions of N-alkyl dihydropyridines. The extreme instability of the unsubstituted dihydropyridines has thus far thwarted the attainment of this end. However, results are promising enough to continue the investigation. A favorable end result would provide a new and versatile synthesis of the morphinan-type analgesics.

Methods: The routine chemical agents and physical instruments.

Part B. Yes

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Part B:

Publications:

Mannich Derivatives of Analgesic Agents by E. M. Fry and Everette L. May. J. Org. Chem. 24, 116 (1959).

Serial No. NIAMD 59
1. Chemistry
2. Analgesics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project title: Comparison of the development and loss of tolerance to the effect of morphine on an analgesic (hot-plate) response and a general behavorial response (swimming) in the rat.

Principal Investigator: Joseph Cochin.

Other Investigator: Conan Kornetsky.

Cooperating Units: NIMH, Dept. of Pharmacology, Boston University School of Medicine, Boston, Mass.

Man years: Total : 1
Professional: 2/3
Other : 1/3

Objectives:

To determine whether the rate of tolerance development on the one hand, and loss on the other hand, is different for two different effects of narcotic drugs.

Methods and Results:

The response of the rat after a test dose of morphine to the analgesic effect is measured by using the hot plate, and at the same time the effect on speed of swimming a circular alley is also measured. The observations were made before, during and after a seventy day period of chronic morphine administration. The effect of this same test-dose on the speed of swimming a circular alley were measured before, during and after a seventy day period of chronic morphine administration. It was found last year that tolerance to the analgesic effect develops more rapidly than loss of sensitivity to the effect of the test-dose on speed of swimming. Continuing this study through 1959, we noted that 262 days after abrupt withdrawal of narcotics, sensitivity to the effect of a 20 mg/kg dose of morphine on swimming speed had returned to initial control values

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but the analgesic response to this test dose is but 45% of the initial control values one year after abrupt withdrawal.

Because of the possibility that the repeated administration of the test dose might be "priming" tolerance and the non-return to initial control drug sensitivity may be due to this, the experiment was redesigned so that this "priming effect" can be studied and its importance evaluated. This part of the study has just gotten under way here and will be under way shortly at Boston University where Dr. Kornetsky is presently located.

Significance to the program of the Institutes:

We believe this to be a contribution to the understanding of the mechanism of the loss of tolerance to narcotic drugs.

Proposed course of the project:

We hope that this newly designed experiment which will isolate and study the "priming effect" of repeated test doses as well as the effect of age and weight on drug response and will answer the question as to whether or not narcotic drug sensitivity is really diminished for such long periods of time after withdrawal. Dr. Kornetsky also plans to do some psychological testing of rats during the periods of addiction and withdrawal which may also throw some light on the problem of tolerance.

Part B included: No.

Serial No. NIAMD - 60
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Study of the Steroidal Alkaloids and Sapogenins

Principal Investigator: Yoshio Sato

Other Investigators: Nobuo Ikekawa and Erich Mosettig

Cooperating Units: None

Man Years
Total: 2 2/3
Professional: 2
Other: 2/3

Project Description:

Objective:— In order to find new, rare and fruitful sources for the production of biologically active steroids, the study of the degradative possibilities of various steroidal alkaloids and sapogenins (particularly solasodine) have continued.

Methods Employed.— The O,N-diacetyl derivatives of solasodine and tomatidine are isomerised by treatment with acids (acetic, pyridine hydrochlorides, etc.) to $\Delta^{20(22)}$ unsaturated pseudo derivatives, oxidised with chromic acid and hydrolyzed with acetic acid to 3β-acetoxy-5,16-pregnadien-20-one and 3β-acetoxy-5α,16-pregnen-20-one respectively. A new tetrahydrosolasodine has been obtained from the aluminum trichloride catalyzed lithium aluminum hydride reduction of solasodine. This has been converted into the hitherto unknown C-22 isomeric solanidan-3-one by oxidation and subsequent reduction. This latest addition completes the set of the four possible C_{22}, C_{25} isomeric solanidanones.

Major Findings.— (1) Conversion of solasodine and tomatidine to the acetates of pregnadienolone and pregnenolone in excellent yields.

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(2) Revelation of the interesting chemistry of the spiroaminoketal system present in the steroidal alkaloids.

Significance.— The degradation of solasodine to pregnadienolone in good yields (65-70%) makes possible the utilization of solanum plants as a commercial steroidal source. In fact Russia has launched on a large scale cultivation of solanum species and industrial conversion of solasodine to biologically active steroids. The knowledge of the manifold chemical interrelationship of the spiroaminoketal system is a definite contribution in the steroidal alkaloid field.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part E. Honors, Awards, and Publications

Publications other than abstracts from this project:

Sato, Y., Ikekawa, N. and Mosettig, E., "Improvement in the Preparation of 3β-Acetoxy-5α,16-pregnen-20-one and 3β-Acetoxy-5,16-pregnadien-20-one from the Steroidal Alakaloids, Tomatidine and Solasodine." J. Org. Chem., 24, 893 (1959).

Sato, Y. and Ikekawa, N., "Preparation of Chenodeoxycholic Acid." J. Org. Chem., 24, 1367 (1959).

Sato, Y., Ikekawa, N. and Mosettig, E., "The Chemistry of the Spiroaminoketal Side Chain of Solasodine and Tomatidine. I. Improved Preparation of 3β-Acetoxy-5,16-pregnadien-20-one and 3β-Acetoxy-5α,16-pregnen-20-one from Solasodine and Tomatidine." J. Org. Chem., accepted for publication.

Sato, Y. and Ikekawa, N., "The Chemistry of the Spiroaminoketal Side Chain of Solasodine and Tomatidine. II. Chemistry of 3β,16β-diacetoxy-20-(2'-Δ^{4-1}-N-acetyl-5'-methyl-tetrahydropyridyl)-5-pregnene." J. Org. Chem., accepted for publication.

Sato, Y. and Ikekawa, N., "The Chemistry of the Spiroaminoketal Side Chain of Solasodine and Tomatidine. III. The Reaction of O,N-diacetylsolasodine in Acidic Media." J. Org. Chem., accepted for publication.

Serial No. NIAMD - 61
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Study of Fecal Steroids

Principal Investigator: Erich Heftmann

Other Investigators: Ekkehard Weiss, Harold K. Miller and Erich Mosettig

Cooperating Units: None

Man Years
Total: 1 1/3
Professional: 1
Other: 1/3

Project Description:

Objective.— To identify steroids in feces.

Methods Employed.— Adsorption and partition chromatography, preparation of derivatives and infrared spectroscopy.

Major Findings.— In addition to the steroids referred to in the 1958 report, we have isolated small amounts of crystalline substances, two of which may be hydroxylated fatty acids and one which is probably a new sterol.

Significance: The nature of the fecal steroid fraction may depend not only on the diet and intestinal flora. Changes in various disease states and in ageing are quite likely and deserve further investigation.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Hoffmann, E., Weiss, E., Miller, K. K., and Mosettig, E., "Isolation of Some Bile Acids and Sterols from the Feces of Healthy Men," Arch. Biochem. & Biophys. 84, 324-41 (1959).

Serial No. NIAMD - 62
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Identification of Acrasin

Principal Investigator: Erich Heftmann

Other Investigators: None

Cooperating Units: Laboratory of Cellular Physiology and Metabolism, National Heart Institute

Man Years
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objective.-- To identify the aggregation hormone in the slime mold.

Methods Employed.-- The methodology developed in the study of fecal steroids was applied to the isolation of the hormone from Dictyostelium discoideum.

Major Findings.-- The identity of acrasin with 22-ethylcholesten-3β-ol was established by chemical means. Other sterols, including ergosterol were found to have acrasin activity.

Significance.-- This is the first demonstration that sterols may have hormonal effects. It is also the first instance in which an organizer has been identified. Sterols may be of general importance in cellular differentiation.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Heftmann, E., Wright, B. E., and Liddel, G. U., "Identification of a Sterol with Acrasin Activity in the Slime Mold," J. Am. Chem. Soc. (accepted for publication).

Serial No. NIAMD - 63
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Biogenesis of Sapogenins

Principal Investigator: Erich Haftmann

Other Investigators: None

Cooperating Units: Division of Biology, California Institute of Technology, Pasadena, California

Man Years
Total: 1/3
Professional: 1/3
Other: -

Project Description:

Objective.— To determine the mechanism whereby steroids are synthesized in plants.

Methods Employed.— Dioscorea tubers are either sliced or homogenized and incubated with radioactive precursors. The labeled products are isolated and identified.

Major Findings.— A method for the isolation of sapogenins has been adapted and applied to two Dioscorea species. D. floribunda has been selected on this basis for the biosynthesis experiments initiated at California Institute of Technology. Dioscorea slices incubated with mevalonic acid convert the latter into 4 radioactive products, none of which is identical with diosgenin. Their identification is in progress.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Heftmann, E., and Moettig, E., "Biochemistry of Steroids," Reinhold Publishing Company, New York. (accepted for publication)

Serial No. NIAMD - 44
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: 1. Determination of Individual 17-Ketosteroids by Gradient Elution Chromatography

2. Adrenocortical Hormones in Rat Adrenal Tumor Tissue

Principal Investigator: David F. Johnson

Other Investigators: Daniel Francois and Erich Heftmann

Cooperating Units: National Cancer Institute - Tumor tissue

Man Years
Total: 2 2/3
Professional: 2
Other: 2/3

Project Description:

Objective:— 1. Development of a quantitative method for the determination of individual 17-keto-steroids in mixtures. 2. Isolation of adrenocortical hormones from samples of transplanted rat adrenal tumor tissue.

Methods Employed.— In the first project a method is being developed for the quantitative determination of individual 17-ketosteroids by a modification of the gradient elution technique with silicic acid columns, developed in this laboratory for adrenocortical hormones. The individual fractions are analyzed by means of the Zimmerman reagent.

The second project is being investigated by the method for adrenocortical hormone determination, i.e. gradient elution with petroleum ether containing increasing amounts of dichloroethane on water impregnated silicic acid columns. The fractions obtained are analyzed by ultraviolet absorption and reduction of blue tetrazolium. Further identification is achieved by paper chromatography.

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Major Findings.— Experiments thus far indicate that alteration of the amount of water, as the stationary phase, on silicic acid columns will permit the separation of the major 17-ketosteroids encountered in biological fluids. Complete separation and quantitative estimation of dehydroepiandrosterone, androsterone, etiocholanalone, 11-ketoandrosterone, and 11-hydroxyetiocholanalone has been achieved. Difficulty has been encountered in the complete separation of 11-ketoetiocholenalone and 11-hydroxyandrosterone but experiments indicate that this can be achieved with the proper conditions.

The second project is being carried out in cooperation with Dr. Katherine Snell of NCI. The tumor tissue being investigated is a transplant from an original spontaneous adrenal tumor in rats. Biological observations indicate that this tumor may be producing adrenocortical hormones. Preliminary fractionations are being carried out to attempt to identify these compounds. Further investigation of the enzyme systems of this tissue with radioactive tracers is planned.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publication concerning report for calendar year 1958, but not reported there:

Johnson, D. F., Francois, D. and Heftmann, E., "Determination of Individual Adrenocortical Steroids in Urine of Pregnant Women," Acta Endocrinol., 32, 8-18, 1959.

Serial No. NIAMD - 65
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The Structure of the Product Obtained from the Lithium Aluminum Hydride Reduction of 22,26-Oxido-$\Delta^{17(20)}$-cholestene-3β,22-diol-16-one.

Principal Investigator: Malcolm J. Thompson

Other Investigator: Erich Mosettig

Cooperating Units: None

Man Years
Total: 1 2/3
Professional: 1
Other: 2/3

Project Description:

Objective.-- Lithium aluminum hydride reduction of 22,26-oxido-$\Delta^{17(20)}$-cholestene-3β,22-diol-16-one had been reported to yield $\Delta^{17(20)}$-22-isoallospirosten-3β-ol. This spirostene would have been an important link in the elucidation of the structure of pennogenin.

Methods Employed.-- Ultraviolet and infrared analysis of the oxidative product of the supposedly spirostene along with analytical data of the original product and derivatives led to the elucidation of the structure of the lithium aluminum hydride reduction product of 22,26-oxido-$\Delta^{17(20)}$-cholestene-3β,22-diol-16-one.

Major Findings.-- It was shown that the lithium aluminum hydride reduction of 22,26-oxido-$\Delta^{17(20)}$-cholestene-3β,22-diol-16-one yields a 22,26-oxido-$\Delta^{17(20)}$-cholestene-3β,16 ξ -diol and not the $\Delta^{17(20)}$-22-isoallospirosten-3β-ol as formerly believed. Analogous results were obtained with the Δ^{5}-series.

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Significance.— Catalytic reduction of the 22,26-amido-$\Delta^{17(20)}$-cholestene-3β,16β-diol has yielded the hitherto unknown 17α-cholestane side chain. Removal of functional groups gives the hydrocarbon, 17-isocholestene. This compound would be of great value as a reference compound where configurational arrangement at C-17 is questionable.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications concerning report for calendar year 1958, but not reported there:

Thompson, M. J., Scheer, I. and Mosettig, E., "The Cholegenins. I. 16,22-Epoxycoprostane-3α,26,27-triol and its Non-identity with Dihydrocholegenin," J. Am. Chem. Soc., 81, 5225-5230 (1959).

Thompson, M. J., Scheer, I. and Mosettig, E., "The Cholegenins. II. Structure of Cholegenin Isocholegenin and Dihydrocholegenin," J. Am. Chem. Soc., 81, 5222-5224 (1959).

Publication concerning report for calendar year 1957 but not reported there:

von Brand, T., McMahon, P., Johnson, T., Thompson, M. J. and Mosettig, E. "Chemical Composition of the Culture Form of Trypanosoma Cruzi," Exp. Parasitol. 8, 171-181 (1959).

Serial No. NIAMD - 66
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The Unsaponifiable Lipids of Taenia taeniaeformis and Moniezia sp.

Principal Investigator: Malcolm J. Thompson

Other Investigator: Erich Mosettig

Cooperating Units: Section of Physiology (Dr. Theodor von Brand), Laboratory of Tropical Diseases, NIAID - 61

Man Years
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objective.— It appeared desirable to reinvestigate more extensively the nonsaponifiable fractions of tapeworms to search carefully for products accompanying cholesterol and finally to characterise all the purified compounds that were isolated.

Methods Employed.— The nonsaponifiable lipids from tapeworms were purified by chromatography. Infrared, ultraviolet and specific rotation analysis were performed on all compounds isolated Further identification was based on direct comparison.

Major Findings.— It was shown that in Taenia taeniaeformis and in Moniezia sp. cholesterol is by far the most prevalent unsaponifiable substance, 98 and 85% respectively. The fact that a search for friedelin in Taenia taeniaeformis was negative strengthens the assumption of Cmelik and Bartl that the friedelin found in Taenia saginata did originate from cork stoppers.

Significance. — The finding of only cholesterol in the tapeworms establishes beyond doubt that the unsaponifiable lipid fraction

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of parasitic worms are not as diversified as in some invertebrate phyla, such as molluscs or sponges.

Part B included Yes

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Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Thompson, M. J., Mosettig, E. and von Brand, T., "The Unsaponifiable Lipids of Taenia taeniaeformis and Moniezia sp." Exptl. Parasitol., accepted for publication.

Serial No. NIAMD - 67
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Study on Hydroxylated Anthrasteroids

Principal Investigator: Osamu Tanaka and J. A. Steele

Other Investigators: Erich Mosettig

Cooperating Units: None

Man Years (calendar year 1959)
Total: 2 2/3
Professional: 2
Other: 2/3

Project Description:

Objective.— This study was undertaken to obtain the hydroxylated anthrasteroids from the corresponding 3-hydroxy-$\Delta^{5,7,9(11)}$-steroids in a pure state.

The chemical structure and the mechanism of the rearrangement are being investigated.

Methods Employed.— Dehydroergosteryl acetate and 3β-acetoxy-$\Delta^{5,7,9(11)}$-cholestatriene were treated with p-toluenesulfonic acid in $CHCl_3$ at room temperature for 15 hours. The product was purified by chromatography and fractional recrystallization as derivatives (p and o-chlorobenzoate, benzhydrobenzoate).

In order to elucidate the reaction mechanism, preparation of $\Delta^{5,7,9(11),22}$-ergostatetraene from dehydroergosteryl acetate maleic anhydride adduct has been undertaken.

Major Findings.— (a) Two isomeric hydroxyanthrasteroids were isolated in ergosterol and cholesterol series. It was found that benzhydrobenzoates of both isomers formed a molecular compound in the case of anthrocholesterol and dihydroanthroergosterol.

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(b) A crystalline keto compound was obtained from one of the isomers of dihydroanthraergosterol by catalytic dehydrogenation. IR and UV spectra showed this keto group was not in conjugation with the aromatic ring.

(c) $\Delta^{5,7,9(11)22}$-Ergostatetraene maleic anhydride adduct was obtained in a good yield.

Significance. -- In contrast to the Bru-Mosettig anthrasteroid rearrangement which is accompanied by dehydration of the 3-hydroxyl group, the new rearrangement with p-toluenesulfonic acid gave two isomers of anthrasteroid with a hydroxyl group.

These compounds seem to be more interesting in the physiological properties compared with the corresponding steroidal compounds.

The formation of an unconjugated keto compound strongly suggested that the hydroxyl group exists in ring A.

Part I included Yes

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Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications concerning report for calendar year 1958, but not reported there:

Burgstahler, A. W. and Mosettig, E., "The Total Synthesis of 11-C-14 Oxygenated Anthracteroids," J. Am. Chem. Soc., 81, 3697-3701 (1959).

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1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The Partial Synthesis of Sulfur-analogs (Positions 11 and/or 9) of Hydroxylated Corticoids and Other Steroidal Hormones

Principal Investigators: Toshio Kawasaki (left NIH 8/14/59)

Other Investigators: Erich Mosettig and Yo Ueda

Cooperating Units: Cancer Chemotherapy National Service Center, NCI -

Man Year (calendar year 1959)
Total: 1 2/3
Professional: 1 1/3
Other: 1/3

Project Description:

Objective.— This study has been commenced with the objective of finding antimetabolites of corticoids and steroidal sex hormones. Such antimetabolites may shed light upon the mechanism of action of antirheumatoid and cancerchemotherapeutic steroidal agents.

Methods Employed.— The methods employed consist mainly in the opening of the 9β,11β- or 9α,11α-epoxide ring with hydrogen thiocyanide, and in the hydrolysis of the resulting thiocyano groups.

Major Findings.— In continuation of the search for sex hormone antimetabolites, Δ^4-androstene-9α-thiocyano-3,11,17-triona has been converted to the corresponding 9α-thiocarbamide and 9α-thiol (9α-mercapto adrenosterone). Similarly, 9α-thiocyanocortisone has been converted to the corresponding 9α-thiocarbamide. A new route to 11β-mercapto corticoids has been opened through the synthesis of 3,9α-epoxy-11β-thiocyano-5β-pregnane-3β,17α,21-triol-20-one 21-acetate.

Part B Included Yes

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Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publication concerning report for calendar year 1958, but not reported there:

Mosettig, E., Biochemistry of Steroids. A Report on Symposium IV., Vol. IV of the Proceedings of the Fourth International Congress of Biochemistry, Vienna, Symposium IV, Pergamon Press 1959, pp. 283-296.

Serial No. NIAMD - 60
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Structure and Stereochemistry of Steviol and Isosteviol

Principal Investigator: Peter Quitt (left NIH 9/30/59)

Other Investigator: Erich Mosettig

Cooperating Units: None

Man Years
Total: 1 2/3
Professional: 1 1/3
Other: 1/3

Project Description:

Objective.-- To elucidate the structure of the aglycone of stevioside.

Major Findings.-- Steviol methylester norketol-allogibberic acid and isosteviol-gibberic acid have superimposable R.D. curves. This establishes the stereochemistry of the six- and five-membered (C/D) ring juncture of steviol and isosteviol. The two epimeric dihydrosteviols obtained by catalytic reduction from steviol and stevioside under different and specific conditions were converted in an eight-step degradation process to (-)α-dihydrokaurene and (-)β-dihydrokaurene.

Significance.-- Important to know the chemical structure of stevioside which is a natural sweetening agent.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Dolder, F., Lichti, H., Mosettig, E. and Gutt, P., "The Structure and Stereochemistry of Steviol and Isosteviol," J. Am. Chem. Soc., accepted for publication.

Serial No. NIAMD - 70
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Infrared Spectroscopic Studies

Principal Investigator: Harold K. Miller

Other Investigators: Richard T. Brown

Cooperating Units: None

Man Years
Total: 2
Professional: 1
Other: 1

Note: All infrared facilities operating Perkin-Elmer Model 21 infrared spectrophotometers in the Laboratory of Chemistry were consolidated in room SB-6, Bldg. 4, during September 1959. This report includes all infrared activities for the calendar year 1959, for the periods before and since consolidation.

Project Description:

Approximately 1100 infrared spectra were made to support investigations in the Laboratory of Chemistry. The power of the infrared method was illustrated in the rapid identification of the material "acrasin" as Δ^{22}-stigmasten-3β-ol, which was confirmed through spectral agreement with authentic material and derivatives. The method of infrared curve synthesis using Lorentzian functions to synthesize the infrared absorption envelope is being investigated in cooperation with Dr. J. Meyer, LED. The 1300-1500 $cm.^{-1}$ region of the spectra of cholestane, androstane, and ergostane, as reported in K. Dobriner's "Infrared Absorption Spectra of Steroids - An Atlas," have been analyzed by the envelope analysis technique, and certain

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Lorentzian components appear to form correlatable sets. These spectra were made with calcium fluoride resolution. This investigation will be continued using the resolution of a prism-grating spectrophotometer and other hydrocarbons in an effort to gain further knowledge concerning the structure of the steroid molecule and its side chains. An attempt to correlate absorptions for morphine type compounds is continuing.

Part B Included No

Serial No. NIAMD, 71

1. Chemistry
2. Independent Unit
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Analytical Services Laboratory

Principal Investigator: William C. Alford (to 11/1/59)
Harold C. Hodges (from 11/1/59)

Other Investigators: Paula H. Pentelius, Evelyn G. Pecka, Elizabeth H. Peth, Byron Kerr, Marie Piper (from July, 1959)

Cooperating Units: Section on Exp. Liver Diseases, NIH, NIAMD

Man Years:
Total: 5 1/2
Professional: 4 1/2
Other: 1

Project Description:

The Analytical Services Laboratory is a service organization providing services for research personnel of the National Institute of Health, and to a limited extent, for persons of other governmental agencies. The scope of this work is summarized as follows:

1. Approximately 10,000 elemental, functional group, and instrumental analyses were performed during the past year. These, with the approximate number of each include: carbon (2175), hydrogen (2175), nitrogen by Dumas, Kjeldahl, and Sevelen techniques (2100), reducing sugar (960), halogens (280), sulfur (260), phosphorus (90), functional groups, such as acetyl, methoxyl, ethoxyl, benzoyl, carboxyl, active hydrogen (85), weight loss, moisture, solvates, ash, etc. (390), micro-weighings (90), metals, such as sodium, copper, barium, zinc, chromium, mercury, cobalt, iron, antimony, etc. (65), optical rotations (75), infrared spectra (1160), selenium (262), miscellaneous (250). Recipients of this service include about 140 research workers of the NIH staff. In addition, analyses are performed for governmental agencies outside NIH, where such service can be given without interfering with the progress of NIH research. During the past year such service was rendered mainly to persons at the Naval Medical Research Institute, Bethesda, Md.

2. During the year the staff has continued services of a special nature to the research team of Dr. Klaus Schwarz (NIAMD) involving a study of the dietary importance of selenium. Over one-half the full-time attention of one member of the staff was required. In addition a considerable amount of work has been performed for Dr. C. H. Keegan, Georgetown University. Dr. Keegan, although employed by NCI is preparing compounds on a contract basis for Cancer Chemotherapy.

3. Considerable time is spent in consultation with research workers concerning problems which they wish to handle in their own laboratories. Methods of approach are discussed, advice is given, and in some cases equipment is provided.

4. Plans have been formulated to set up a chromatography service laboratory. An analyst has been obtained for this work, and this service will be offered when space is available.

5. The infra-red service laboratory is a part of the Analytical Services Unit. Details of their work are described in a separate report.

Part B included: No

Project No. [illegible]
1. Pathology & Histochemistry
2. Histochemistry
3. Bethesda

Part A

Principal Investigators: R. D. Lillie

Other Investigators: None

Cooperating Units: None

Man Years (Calendar Year 1959)

Total:	3
Professional:	1
Other:	2

Project Description:

Work on the histochemical reactions of the carcinoid tumors of the human gastrointestinal tract was completed, the pertinent literature of some 150 titles reviewed critically and the report has been accepted for publication in the American Journal of Pathology.

Work on the identification of the enterochromaffin substance has continued. In view of the reported isolation of 5-hydroxytryptamine in the hypertonic sucrose centrifugate, the sucrose bromide was changed to histologic use, but it did not prove possible to demonstrate indole reactions in enterochromaffin cells.

Detailed study of the azo coupling reaction of enterochromaffin has been made with a considerable range of stable and fresh diazonium. Reversible oxidation blockade of the azo reaction has been demonstrated. The product of the chromaffin reaction of enterochromaffin is probably a carboxylic acid. This would appear to demand ring cleavage. Further study is required on this point. Total elucidation of the enterochromaffin substance has been demonstrated. The intermediate of this reaction is not yet established, though an indole diphenol or a tryptophan seem to be indicated. Model preparations of the related reactive compounds have been made in study, with formaldehyde alkylation. These have been subjected to the various reactions used for identification of enterochromaffin and the data are being studied.

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

S. S. Spicer and R. D. Lillie: Saponification as a means of selectively reversing the methylation blockade of tissue basophilia. J. Histochem. Cytochem. 7: 123-125, 1959.

G. G. Glenner and R. D. Lillie: Pepsin release of guinea pig entero-chromaffin substance. J. Histochem. Cytochem. 7: 204, 1959.

R. D. Lillie: Preferred common names, formulae, colour index references and synonyms of stable diazonium salts used in histochemistry. J. Histochem. Cytochem. 7:282-284, 1959.

G. G. Glenner and R. D. Lillie: Observations on the diazotization coupling reaction for the histochemical demonstration of tyrosine, metal chelation and formazan variants. J. Histochem. Cytochem. (In Press)

G. G. Glenner and R. D. Lillie: Observations on the diazotization-coupling reaction for the histochemical demonstration of tyrosine: Metal chelation and formazan variants. J. Histochem. Cytochem. (In Press)

R. D. Lillie and J. G. Henson: An attempt at demonstration of an indolic substance in enterochromaffin cells by use of hypertonic sucrose solution. J. Histochem. Cytochem. (In Press)

Honors and Awards relating to this project:

Dr. R. D. Lillie was elected President of the Biological Stain Commission, 1959.

Serial No. NIAMD-72
1. Pathology & Histochemistry
2. Histochemistry
3. Bethesda

Part A

Project Title: Histochemical demonstration of oxidative and proteolytic enzymes in normal and pathologic tissue

Principal Investigator: Dr. G. G. Glenner

Other Investigators: None

Cooperating Units:

NIAMD-LC:	Dr. I. A. Cohen	NIAMD-LC
NIAMD-CE:	Dr. J. B. Field	NIAMD-LEC
NCI-B:	Drs. B. Horta & D. Kellogg	NCI-BDBC
NIDR-LMP:	Dr. H. S. Mandsbow	NIDR-L
NHI-LCB:	Dr. H. H. Weissbach	NHI-LCB

University of Pennsylvania, Henry Phipps Institute:
Dr. A. Dannenberg, jr.

Man Years (Calendar Year 1959)

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

Project Description:

Extensive studies on steroid oxidative enzymes have been [illegible] taken in an attempt to evaluate known morphologic changes in [illegible] animal and biochemical findings on the basis of individual cell function. Using the information obtained from the biochemical experiments described by Villee and Talalay pertaining to the [illegible] of an estrogen-sensitive dehydrogenase system in placenta, [illegible] the first histochemical demonstration of both a NAD and NADP [illegible] 17 beta-steroid dehydrogenase in placenta. Reasons for the basis of the difference in localization of these two enzymes [illegible] Linked systems it was possible to confirm the localization of the [illegible] enzymes of specific [illegible] to indicate the possibility that a [illegible] was present in a single [illegible] of the placenta.

Based on the original [illegible] the enzyme in the [illegible] study of this enzyme in [illegible] neoplastic human tissues [illegible] certain tumor types was [illegible]

The majority of epithelial tumors revealed high enzymatic activity in the invaded stroma only. Aminopeptidase activity in the stroma adjacent to invasive tumors was direct evidence of proteolysis and was unrelated to inflammatory or fibroblastic reaction in tumor stroma, thereby indicating that a prominent mechanism of tumor invasion is by the proteolytic destruction of the stromal compartment. However, this did not imply that all invasive tumors showed increased stromal aminopeptidase activity. It is indicated, rather, that different tumor types (notably sarcomas) invade tissue by a mechanism other than that demonstrated by this histochemical technique. It was also evident from the above study that increased stromal aminopeptidase activity was probably related either to the activation of the enzyme by a metabolic product of the invasive tumor or by an immune response.

Further studies on the presence of other enzymes in the stroma of invasive tumors is being undertaken in an attempt to determine whether specific tumors invade on the basis of tumor specific proteolytic or hyaluronidase-like enzymatic solution of adjacent stroma. In this regard a histochemical substrate specific (in a biochemical system) for trypsin was synthesized. Though the enzyme hydrolysing this substrate was not evident in tumor stroma, it was present specifically in the mast cells of certain species. This initial demonstration of a species-limited trypsin-like enzyme in mast cells gives concrete definition to an enzyme capable of producing intracytoplasmic proteolysis. An enzyme of this type had been previously implicated by Unger in the release of intracytoplasmic granule substance (histamine and heparin) from the mast cell following chemical injury.

A continuation of work on the localization of specific amino acids by new histochemical techniques has revealed the presence of high concentrations of protein-bound tyrosine localized almost exclusively in the alpha cells of the anterior hypophysis. Evaluation of this histologic finding as it relates to pituitary hormonal production is in its preliminary stages.

Part B included

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

G. G. Glenner, M. S. Burstone and D. B. Meyer: A study of aminopeptidase activity in the stroma of neoplastic tissue with a comparison of histochemical techniques. J. Nat. Cancer Inst. 23: 857-874, 1959.

G. G. Glenner and R. D. Lillie: Pepsin release of guinea pig entero-chromaffin substance. J. Histochem. Cytochem. 7: 204, 1959.

G. G. Glenner and R. D. Lillie: Observations on the diazotization-coupling reaction for the histochemical demonstration of tyrosine: metal chelation and formazan variants. J. Histochem. Cytochem. 7: 416-422, 1959.

G. G. Glenner: A nitrosophenol reaction for tyrosine and related compounds in tissue sections. J. Histochem. Cytochem. 7: 423-424, 1959.

G. G. Glenner and L. A. Cohen: The histochemical demonstration of a species-specific enzyme trypsin-like in mast cells. Nature (In Press).

G. G. Glenner and H. E. Bagdoyan: Tyrosine localization in hypophyseal alpha cells. J. Histochem. Cytochem. (In Press)

R. D. Lillie and G. G. Glenner: The histochemical reactions of the carcinoid tumors of the gastrointestinal tract. Am. J. Path. (In Press).

G. G. Glenner, H. Weissbach and B. Redfield: The histochemical demonstration of enzymatic activity by a nonenzymatic redox reaction. Reduction of tetrazolium salts by indolyl-3-acetaldehyde. J. Histochem. Cytochem. (In Press)

Honors and Awards relating to this project: None.

[redacted]
(Calendar Year 1959)

Serial No. [redacted]-74
1. Pathology & Histochemistry
2. Histochemistry
3. Bethesda

Part A

Project Title: Studies on the histochemical reaction of mucoproteins

Principal Investigator: S. S. Spicer

Other Investigators: None

Cooperating Units:

C NP:	Dr. Richard L. Simon	NCI-511
A LPT:	Dr. L. Weston	NIAMD-98
C NPM:	Dr. S. E. Wolman	NCI-906
C LP:	Dr. T. B. Dunn	NCI-510

Man Years (Calendar year 1959)

Total: 2-1/2
Professional: 1
Others: 1-1/2

Project description:

The primary interest during the past year centered histochemical methods of localizing, differentiating and further characterizing rodent mucopolysaccharides. Basically, this project involved confirmation of results yielded by current staining procedures with results obtained by radioautographic localization of sulfated moieties with $Na_2S^{35}O_4$. The autoradiographic studies employing S^{35} were done in collaboration with Dr. R. E. Simon of the National Cancer Institute. By this means it was shown that a number of alcian blue reactive and periodic-Schiff reactive acid mucopolysaccharides lack sulfate esters. Chemically a mild methylation procedure differentiates the carboxyls in sulfated acid moiety blocking their basophilia. Another feature of these polymers is their capacity to combine with trivalent iron at pH 1.5 but not at pH 3.5. A third characteristic which differentiates sulfated acid moiety from those with sulfate esters is their lesser affinity for ferric iron. This was demonstrated by employing the basic dye alcian blue at pH 1.0 and 2.5. Whereas sulfated acid moiety stained at pH 1.0 and 2.5, sulfate ester acid moiety stained only at pH 1.0. Mucin of one particular [redacted] (rat and [redacted]) of the sulfated acid moiety has been found [redacted] following specific digestion with [redacted] and by specific [redacted] pigment and digestion with [redacted]

for the first time identifies sialomucins histologically, sialic acid containing mucins of the mouse have been localized in the sublingual glands, laryngotracheal glands, thyroid cyst contents, and vaginal surface epithelium during pregnancy. The basophilia of sialomucins in the rat resists digestion by the available sialidase preparations; but the metachromasia of the guinea pig sublingual glands succombs to such treatment. The metachromasia of the follicular fluid in certain sialic acid rich thyroid cancers in the rat, examined in collaboration with Dr. S.H. Wollman of the National Cancer Institute, also disappears following sialidase digestion.

As shown by comparison of alcian blue staining with S^{35} radioautographs and azure A staining at low pH, many highly sulfated mucins, including those of mast cells and cartilage lack alcian blue reactivity. However, mast cells in some areas in mice (like certain epithelial sulfated mucins) stain with alcian blue and show relatively weak metachromasia indicating functional depletion of sulfated mucopolysaccharides. Investigation into the distribution of alcian blue reactive mast cells has shown that they are usually intimately associated with numerous phagocytes laden with stainable iron and lipofuscin. These mast cells and phagocytes increase concurrently with age in association with the appearance of intracytoplasmic iron positive granules and/or lipofuscin bodies in certain epithelial cells. The significance of the latter observations is under investigation currently from the point of view that mast cell mucins may play a role in the phagocyte disposal of wear and tear pigments and products of cellular degeneration.

An additional project currently under investigation relates to the chemical nature of certain hematoxylin stained bodies observed in the adrenal gland by Dr. Thelma Dunn of the National Cancer Institute. In collaboration with Dr. Dunn it has been found that these bodies consist of a reducing substance related to, but not identical with the catechol amines as demonstrated by the chromaffin reaction. The presence of these bodies in adrenal tumors and of histochemically similar granules in cortical cells in cases of Cushing's syndrome has been demonstrated.

Part B included

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

S. S. Spicer & R. D. Lillie: Saponification as a means of selectively reversing the methylation blockade of tissue basophilia. J. Histochem. Cytochem. 7:123-125, 1959.

S. S. Spicer: A correlative study of the histochemical properties of rodent acid mucopolysaccharides. J. Histochem. Cytochem. (In Press).

S. S. Spicer, Helen J. Burtner and R. L. Suarm: Comparison of basophilia with S^{35} label in normal and methylated mucopolysaccharides. (In Press)

S. S. Spicer and L. Warren: The histochemistry of sialic acid conteining mucoproteins. J. Histochem. Cytochem. (In Press)

S. S. Spicer and D. B. Meyer: Histochemical Differentiation of acid mucopolysaccharides by combined aldehyde fuchsin alcian blue staining. (Am. J. Clin. Path. In Press)

Honors and Awards relating to this project:

None

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD-75
1. Pathology & Histochemistry
2. Histochemistry
3. Bethesda

Part A

Project Title: Studies on renal structure and function

Principal Investigator: J. B. Longley

Other Investigator: M. B. Weiss (Student Scientist)

Cooperating Units: NCI-LP: Dr. W. G. Banfield

Man Years (Calendar Year 1959)

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

Project description:

a) Studies of the countercurrent vascular bundles found in the medulla of mammalian kidneys have been continued, with the aid of the electron microscope. The fine structure of the afferent and efferent capillaries has been observed. The afferent vessels (branches of the efferent arterioles of the juxta-medullary glomeruli) reveal a thick endothelium in which the cells overlie one another extensively. These are the cells which in the rat have previously been found to possess strong esterase activity. The basement membrane is complete, and only occasional pericapillary investing cells are seen. Similar capillaries have been described in cardiac and skeletal muscle, in lung, in elastic tissue, and in the nervous system. The efferent vessels are lined by a delicate fenestrated endothelium similar to that already described in glomerular and interlobular capillaries in the mammalian kidney. These capillaries also possess a continuous basement membrane; investing cells have not been seen. Between the two types of capillaries moderate interstitial spaces occur. The structural details seen in this study raise the question which has already been asked in previous histochemical studies; whether it is not likely that these vascular structures do not have important functions in addition to permitting osmotic equilibration between the two opposing streams of blood they carry. As studies continue clues to this problem may be found.

b) The distribution of glutaminase I in the kidney of the rat has been determined. It has been concluded that the main sites of renal glutaminase I activity in the rat are in the convoluted part

of the proximal tubule and in the straight segment of the distal tubule. It has been shown that the amount of glutaminase I activity in the renal papilla of the rat is insufficient to account for the observed rates of ammonia excretion, and it is therefore indicated that this enzyme is not the direct agent of release of urinary ammonia.

c) The handling of the dye chlorophenol red by the proximal tubule of the rat kidney has been investigated. In vivo, or at low perfusion pressure, or under urinary stop flow conditions, dye concentrates in the convoluted segment. In vivo the dye concentrates in the straight segment. This difference correlates with tubular flow of urine, and the results show that tubular excretion of the dye takes place only in the convoluted segment, and that part of the excreted dye is actively reabsorbed in the straight segment. This is the first direct demonstration of a functional difference in these two segments of the mammalian renal tubule.

Part B included.

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

J. B. Longley, W. G. Banfield, and D. C. Brindley. Structure of the rete mirabile in the kidney of the rat as seen with the electron microscope. J. Biophys. Biochem. (In Press).

M. B. Weiss and J. B. Longley: Renal glutaminase I distribution and ammonia excretion in the rat. Am. J. Physiol. (In Press)

Honors and Awards relating to this project:

Dr. J. B. Longley was elected Trustee of the Biological Stain Commission in April 1959.

Dr. J. B. Longley was appointed Society Representative to the Biological Stain Commission by the Histochemical Society at its annual meeting in Atlantic City in 1959.

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD - 76
1. Pathology and Histochemistry
2. Histochemistry
3. Bethesda

Part A

Project Title: Histologic studies of genes and chromosomes in normal and pathologic conditions.

Principal Investigator: Dr. J. H. Tjio

Other Investigators: None

Cooperating Units: None

Man Years (Calendar Year 1959) Dr. Tjio arrived on October 15, 1959.

Project description:

Studies on the karyotype of man in relation to 1) the localisation of specific human genes and chromosomal aberrations in subjects with hereditary defects; 2) the identification of chromosomal sex constitution of individuals with varying degrees of clinical hermaphroditism; 3) the nature of malignancy. A study of the sex chromosomes during male meiosis (spermatogenesis) in man and on the nature of malignancy from a chromosal point of view are also in progress.

No Part B. etc..

No Honors and Awards relating to this project.

Individual Project Report
Calendar Year 1959

Serial No. NIAMD - 77
1. Pathology & Histochemistry
2. Histochemistry
3. Bethesda

Part A

Project Title: Histochemical studies on phosphorylating enzymes

Principal Investigator: T. Takeuchi

Cooperating Units: None

Man Years: Dr. Takeuchi arrived as a Visiting Scientist on October 30, 1959.

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIMAD - 78
1. Pathology & Histochemistry
2. Histochemistry
3. Bethesda

Part A

Project Title: Histochemical studies of neuromelanins

Principal Investigator: Dr. H. Yamada

Other Investigator: None

Cooperating Units: None

Man Years (Calendar Year 1959)

Total:	2
Professional:	1
Other:	1

Project Description:

An examination of the histochemical staining of neuromelanins in the locus caeruleus and substantia nigra of human and monkey brains was made. This revealed that neuromelanins fail to exhibit a reactive sulfate or sulfonic acid grouping as seen in cutaneous melanin. The presence of eosinophilic granules in the nerve cells of the locus caeruleus are much more highly developed than those of the substantia nigra. These eosinophilic granules, which probably represent phenolic protein substances, decrease with age and appear to have a relationship to the formation of melanin pigments in these sites.

Part B. Honors, Awards, and Publications: None

Serial No. NIAMD - 79
1. Pathology & Histochemistry
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Pathology of choline deficiency in germ-free rats, Trichomonas vaginalis infection in mice and allergic encephalitis in guinea pigs.

Principal Investigator: L. L. Ashburn

Other Investigators: David L. Beaver, Ernest G. McDaniel, Floyd S. Daft, Stanley Levenson, George Hottle and Lucy Reardon.

Cooperating Units: LME-NIAMD Department of Germ-free Research-NRAIDS
DBS LPP-NIAID

Man Years (calendar year 1959)
Total: 2
Professional: 1
Other: 1

Project Description:

Preliminary studies in germ-free rats indicate that choline deficiency develops in these animals as readily as in conventional animals. Fat accumulates in appropriate locations within liver lobules, ceroid is formed (cod liver oil in diet) and phagocytosed, liver cells degenerate, lobules become distorted and connective tissue is greatly increased in amount, appearing in trabeculae separating remnants of lobules or largely replacing parenchyma in main lobes near portal hilatus and the papillary lobes. This study will be extended and include an evaluation of factors (protein adequacy, dietary supplements) influencing the localization of fat within the liver lobule.

Continued studies of allergic encephalitis in guinea pigs have aimed at determining the earliest age at which the "allergin" appears in the rabbit brain. Such information was needed in connection with an attempt (cooperative study - DBS) to produce a rabies vaccine from rabbit brain that will not produce allergic encephalitis when tested in guinea pigs using the usual adjuvant. The data from these studies show that the "allergin" is variably present in brain tissue of

rabbits 7 to 9 days old but not in those under 7 days; also that infection with rabies does not alter the time when the allergin appears, and that a vaccine may be made from such young rabbits. Study will continue on the latter point.

In search for in vivo method of evaluating agents toxic for Trichomonas vaginalis a strain has been found (Laboratory of Protozoology, NIAID) that produced severe lesions in mice following intraperitoneal injection. Organisms proliferate here, induce inflammation in the omentum, mesentery, and areolar tissue about the kidney, pancreas, and portal fissure. Invasion of the liver occurs from the latter location as well as directly through capsule. Once the liver is involved, the organisms rapidly increase in number, move forward on a broad front, leaving behind only necrotic tissue. The protozoa forming this "moving wall" are filled with glycogen (red with P.A.S. technique) and suggest a "red tide". Liver cells immediately ahead of the parasite wall show little change. Studies will be made in an attempt to determine how the trichomonads destroy the liver cells.

Part B included: No.

Serial No. NIAM - 20
1. Pathology & Histochemistry
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Studies on dietary hemosiderosis, on sarcoidosis, and on pneumocystis infection in man. Experimental studies with pneumocystis in rats and mercurial poisoning in cats.

Principal Investigator: Gert L. Laqueur

Other Investigators: Leon Jacobs, L. T. Kurland, O. Mickelsen

Cooperating Units: NID-NIAID E-NIADB LMS-NIAID

Man Years (calendar year 1959):
Total: 2
Professional: 1
Other: 1

Project Description:

The study of the relative incidence of degenerative cardiovascular diseases among Japanese of Hiroshima has been completed in its essential aspects and will be published after arrival of certain outstanding data from Japan.

Pathologic examination of surgical and autopsy materials from the PHS Indian Hospitals and certain Federal Institutions has been continued and several studies have been initiated from this material. Among them, the problem of dietary hemosiderosis among Indians is of interest in view of the relative frequency with which severe forms of chronic liver disease perhaps dietary in nature are seen in this particular material.

Cases of sarcoidosis among full-blooded Indians including a follow-up correspondence with the respective Indian Hospitals were reviewed. This has been done preparatory to participation in an International Conference on sarcoidosis next year. Along this line, preliminary arrangements have been made to participate in an epidemiologic study of sarcoidosis among the Pima-Papago Indians.

An outbreak of a pulmonary infection with high mortality occurred in a Korean orphanage early in 1959. Material sent here was diagnosed as plasmocytic interstitial pneumonia associated with overwhelming infections with pneumocystis carinii. A summary of the many problems in this disease was recently submitted, and it was pointed out that experimental studies had been initiated designed to investigate such phases as taxonomy, pathogenesis, antigenicity, and responses to chemotherapy and/or antibiotics.

The first objective was to provide large numbers of living organisms. Fresh human material being difficult to obtain, the attempt was made to utilize the rat as a source because pneumocysts have been found occasionally in various rat colonies. By using cortisone and antibiotics, large numbers of organism were found after several months. Having established a source for pneumocysts, the second objective was to obtain *in vitro* growth of the organisms. These experiments are presently in progress in collaboration with Dr. Jacobs of NIAID.

The outbreak of a neurologic disorder of man, cats, and birds with a high mortality was observed in a Japanese fishing village on Minamata Bay. The disease according to reports results from consuming fish caught in this bay into which a factory empties its refuse. Organic mercury compounds have been implicated as the agent responsible for the development of the disease. A collaborative study with the Epidemiology Branch of the NINDB and the LNE of NIAMD is under way. Pathologic studies on cats fed seafood from the Japanese bay thus far have been unremarkable. In those which were affected, severe organic alterations were found in the cerebellum and basal ganglia. Experiments are now in progress and include for comparison studies on cats fed organic mercury compounds.

Part B included: No

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Studies on high altitude, experimental [illegible] and other cardiac lesions, arteriosclerosis, [illegible] endocarditis and glomerulonephritis, catecholamines, adrenergic and ganglionic blocking agents, monoamine oxidase inhibitors, and fat mobilization and deposition in the liver.

Principal Investigator: Benjamin Highman

Other Investigators: P. D. Altland, B. B. Brodie, W. M. Butler, V. H. Cohn, Jr., H. M. Maling, J. Roche, and E. C. Thompson.

Cooperating Units: LPB-NIAMD (Altland and Thompson) NIAMD-104
LCP-NHI (Maling, Brodie, Butler, and Cohn) NHI-200

Man Years (calendar year 1959):
Total: 1
Professional: 1
Other: None

Project Description:

Studies with Drs. Altland and Roche on the effects of repeated daily exposures to 30,000 feet on normal dogs and on dogs [illegible] valvular deformities were completed. In addition to valvular thickening and repetations, renal infarction and hemosiderosis, and other changes previously described in rats exposed to 25,000 feet, the dogs often developed medial-intimal arteriosclerotic intimal plaques involving the aorta and occasionally the coronary arteries; these lesions are attributed to hypoxia. This experimental reproduction of lesions resembling human arteriosclerosis may prove useful in elucidating the genesis of arteriosclerosis in man.

Another study with Drs. Altland and Roche was completed on the treatment of experimental staphylococcal endocarditis. This was produced by an intravenous injection of a culture of Staphylococcus aureus in dogs rendered highly susceptible by surgically-induced aortic insufficiency. Endocarditis could be prevented by a

single intravenous injection of penicillin given within 6 hours after injecting the bacteria. If treatment was delayed 24 hours or longer, the symptoms often recurred after cessation of treatment. The endocarditis was arrested by treatment of relapses, but the valves often became thickened and deformed and acute animals died from acute heart failure due to valvular insufficiency. A diffuse proliferative glomerulonephritis, which developed in nearly all dogs given delayed treatment, persisted despite therapy. There was evidence that a chronic glomerulonephritis may be a sequel to this proliferative glomerulonephritis. These findings emphasize the importance of prophylactic treatment in susceptible individuals and of early and adequate treatment of human endocarditis. They support the thesis, recently questioned, that a chronic glomerulonephritis is a sequel to an acute nephritis. Our experimental method should prove a useful tool in studying staphylococcal infections resistant to antibiotic therapy and in the study of glomerulonephritis.

Studies with Dr. Altland on the effects of high altitude on cholesterol-fed rabbits are nearing completion. Studies were begun with Dr. Altland on changes in blood chemistry induced by exposure of dogs to high altitude, on possible changes in immunity and antigenic response in rats exposed to high altitude, and on the effect of various drugs on the susceptibility to endocarditis of normal rats and rats exposed to high altitude.

In studies with Dr. Kuling, it was found that large doses of catecholamines produce a marked elevation in serum lactic dehydrogenase in addition to the previously reported marked myocardial fatty changes and elevations in serum transaminases and alkaline phosphatase. Such changes, excepting the rise in serum alkaline phosphatase, are reduced or prevented by the adrenergic blocking agent, phenoxybenzamine. However, phenoxybenzamine does not prevent a similar rise in serum enzyme levels that occurs after myocardial infarction in dogs following coronary ligation, nor does it prevent the fatty changes in the myocardium bordering the infarct.

A study was made to test the concept of Harris and Pisteni that the ventricular tachycardia resulting from myocardial infarction is due, at least in part, to epinephrine and norepinephrine, which are liberated from the necrotic myocardium and which may act upon the functional cells bordering the infarct. The myocardium was depleted of norepinephrine by administering reserpine to dogs before coronary ligation. This did not prevent the ventricular tachycardia, the fatty changes around the infarct, and the rise in serum enzyme levels. These findings do not support the concept of Harris and Pisteni.

A study with Butler, Maling, and Brodie concerns an increase in neutral fat or triglyceride content of the liver of rats induced by carbon tetrachloride (1.5 cc/kg s.c.), ethanol (6 gm/kg orally), or ethionine (750 mg/kg i.p.). It was found by chemical and histologic methods (staining for fat with oil red O) that the increase in fat was largely prevented by prior administration of adrenergic blocking agents. This project should increase our understanding of the processes involved in triglyceride mobilization and deposition and may give some clues to the nature of hepatic cirrhosis and atherosclerosis.

Another study with Drs. Maling and Spector concerns the administration of various monoamine oxidase inhibitors to dogs, cats, rabbits, and squirrel monkeys. Some of these compounds produce marked changes in behavior and marked neurologic symptoms. These findings are correlated with changes in the levels of serotonin and norepinephrine in various parts of the nervous system and with pathologic changes. This study may elucidate the mode of action and possible toxicity of some of these compounds. This study is important because some of these compounds are widely used by psychiatrists and in the treatment of hypertension.

Serial No. NIAMD-81

PHS-NIH
Individual Project Report
Calendar Year 1959

Part E: Honors, Awards, and Publications

Participated in the Symposium on "The Catecholamines in Cardiovascular Pathology" which was held at the University of Vermont, College of Medicine, August 23-26, 1959.

Participated in Meeting of Joint Committee On Aviation Pathology which was held at the Armed Forces Institute of Pathology, Washington, D. C., on November 6, 1959.

Publications other than abstracts from this project:

Webster, S. H., Rice, M. E., Highman, B., and Stohlman, E. R.: The Toxicology of Potassium and Sodium Iodates II. Subacute Toxicity of Potassium Iodate in Mice and Guinea Pigs. Toxicology and Applied Pharmacology 1: 87-96, 1959.

Highman, B., Maling, H. M., and Thompson, E. C.: Serum Transaminase and Alkaline Phosphatase Levels after Large Doses of Norepinephrine and Epinephrine in Dogs. Am. J. Physiol. 196: 436-440, 1959.

Maling, H. M. and Highman, B.: High Altitude Tolerance of Normal Dogs and Dogs with Myocardial Infarcts. Am. J. Physiol. 196: 507-511, 1959.

Maling, H. M., Cohn, V. H., Jr., and Highman, B.: The Effects of Coronary Occlusion in Dogs Treated with Reserpine and in Dogs Treated with Phenoxybenzamine. J. Pharmacol. & Exper. Therap. 127: 229-235, 1959.

Altland, P. D., Highman, B., and Roshe, J.: Effects of Altitude on Dogs with Valvular Heart Disease. A. M. A. Arch. Path. 68: 475-486, 1959.

Highman, B., Altland, P. D., and Roshe, J.: Staphylococcal Endocarditis and Glomerulonephritis in Dogs. Effects of Treatment with Penicillin and Streptomycin. Circulation Research 7: 982-987, 1959.

Maling, H. M., Highman, B., and Thompson, E. C.: Some Similar Effects After Large Doses of Catecholamines and Myocardial Infarction in Dogs. Paper presented at Symposium on "The Catecholamines in Cardiovascular Pathology" held at Burlington, Vermont, August 23-26, 1959 and paper submitted at request for publication in the American Journal of Cardiology.

Altland, P. D., and Highman, B.: Effects of High Altitude on Cholesterol Fed Rabbits. Production of Severe Pulmonary Atherosclerosis with Calcification. A.M.A. Arch. Path. In press.

Serial No. NIAMD-82
1. Pathology & Histochemistry
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Study of mechanisms involved in infectious diseases.

Principal Investigator: Edwin M. Lerner II

Other Investigators: Leon Sokoloff, Robert R. Williams, Kurt J. Bloch, Joseph J. Bunim, Robert T. Habermann, Sheldon Dray

Cooperating Units: LPB-NIAMD-109 A&R-NIAMD - LAB-DRS 125C LI-NIAID - 55

Man Years (calendar year 1959):
Total: 2
Professional: 1
Other: 1

Project Description:

The pathogenesis of experimentally induced arthritis in rats by infection with Streptobacillus moniliformis has been defined in detail. The lesions have been shown to develop as a primary osteomyelitis, with secondary extension into the periarticular soft tissues, or into the joint from either of the two preceding foci. The similarity of this infectious process to other so-called infectious arthritides has been described. Bacteriological and immunological studies have been correlated with a detailed time study of this process.

The infectivity of the currently employed strain has been explored in mice which had been raised so as to be free of natural infection with Streptobacillus or with pleuropneumonia-like organisms. The incidence of gross joint lesions has been as great in mice as was observed in rats, and the gross evidence of inflammation persisted for much longer periods of time in mice. The microorganism was found to be lethal for mice after intravenous, intraperitoneal, or subcutaneous injection, with decreasing rate of mortality in that order of routes of inoculation. Gross joint lesions appeared in mice as early as 24 hours after injection, and have persisted for as long as three months.

The serological reactions for rheumatoid arthritis in the infected rats, namely; the sensitized sheep cell hemagglutination reaction and the bentonite flocculation test, have been shown to be an immunologic response to antigenic stimulation, independent of active infection or joint inflammation. Rabbits immunized with formalin-killed antigens prepared from S. moniliformis developed high bentonite flocculation test titres, ranging up to 1: 1024. Attempts to characterize the protein fraction involved in this immune response in rabbits indicated that the responsible factor in rabbit serum differed from that in human rheumatoid arthritis serum by ultracentrifugation behavior and by agar gel precipitation tests. The rabbit factor had the characteristics of a small molecule by ultracentrifugation, thus differing from the human rheumatoid factor. Agar gel precipitin experiments indicated that the immunized rabbit serum contained an antibody to human gamma globulin. Although the microorganism was routinely cultivated in medium containing human ascitic fluid, this substance had been found inactive in control experiments involving both these serological reactions. Further exploration of the role of human ascitic fluid included adaptation of the microorganism to growth in semi-synthetic medium, and finding that such cultures were non-immunogenic in the tests involved. Human ascitic fluid alone, or in combination with bentonite particles or with S. moniliformis grown in the absence of ascitic fluid was non-stimulatory. This indicated that the microorganism either concentrated or modified human gamma globulin present in the medium so that it became antigenic, and the similarity of this process with the elaboration of haptenogenic materials from culture media by other microorganisms has been defined.

Part B included: Yes

Serial No. NYNMm - 82

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Lerner, Edwin M. II, and Scholoff, Leon: The Pathogenesis of Bone and Joint Infection Produced in Rats by Streptobacillus moniliformis. Am. Arch. Path. 67, 364-372; 1959.

Lerner, Edwin M. II, Bloch, Kurt J., and Williams, Robert R., Jr.: "Rheumatoid" Serological Reactions in Experimental Animals. II. Bentonite Flocculation Test in Rats with Experimental Arthritis. Arthritis and Rheumatism. In Press.

Lerner, Edwin M. II, Bloch, Kurt J., and Williams, Robert R., Jr.: "Rheumatoid" Serological Reactions in Experimental Animals--The Sensitised Sheep Cell Hemagglutination Reaction and Bentonite Flocculation Test in Rats with Experimental Arthritis. Second Pan-American Congress on Rheumatic Diseases, June, 1959.

Lerner, Edwin M. II: Arthritis Caused by Streptobacillus moniliformis and Pleuropneumonia-like Organisms in Small Rodents. Conference on the Comparative Pathology of Arthritis and Rheumatism. Laboratory Investigation. In Press.

Lerner, Edwin M. II: Pathology of Acute and Chronic Brucellosis in Experimentally Infected Guinea Pigs. Conference on the Comparative Pathology of Arthritis and Rheumatism. Laboratory Investigation.

Lerner, Edwin M. II: Morphology and Classification of the Pleuropneumonia-like Organisms. Summary of Session Chairman. New York Academy of Sciences Conference on Biology of the Pleuropneumonialike Organisms. Annals New York Academy of Sciences. In Press.

Serial No. NIAMD - 83
1. Pathology & Histochemistry
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Morphologic and histochemical variations in the preputial glands, endocrine system, and internal genitalia of the conventional and germ-free rat, as influenced by hormones, vitamins, bacteria, and tissue fixation.

Principal Investigator: David L. Beaver

Other Investigator: Ernest G. McDaniel

Cooperating Unit: LME-NIAMD - 3

Man Years (calendar year 1959):
Total: 1
Professional: 1
Other: None

Project Description:

It has been shown that the rat preputial gland is a "dioxine" organ and any histochemical or endocrinological study must take this into consideration. A method has been developed for staining the two secretory products simultaneously and is now being used, along with various histochemical procedures, to assess the effect of various hormones on the gland. Concommitantly vitamin A deficiency is being studied in the conventional rat from an endocrinologic point of view, and in the germ-free rat in order to determine the importance of infection in the production of squamous metaplasia. In addition, the relationship of normal bacterial flora to the neutrophilic vaginal exudate of the murine estrous cycle is being evaluated. It is hoped that the information obtained from these studies will lead to a better understanding of experimentally induced tissue changes.

I am also collaborating with others in an experiment concerned with the role of vitamin B_{12} and choline on the production of dietary cirrhosis in the germ-free rat (see report by Dr. L. L. Ashburn).

Part B included: Yes.

Serial No. NIAMD-83

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Beaver, David L.: "Der Einfluss verschiedener Fixierungsmittel auf das Strukturbild der Präputialdrüsen der Ratte": To be published in Zeitschrift für Zellforschung und Mikroskopische Anatomie.

Serial No. NIAMD-84
1. Pathology & Histochemistry
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Preparation of stained tissue section for investigation and diagnostic purpose.

Principal Investigator: Mr. Roy Reed - Head, tissue preparation laboratory.

Other Investigator: None

Cooperating Unit: None

Man Years (calendar year 1959):
Total: 7*
Professional: 0
Other: 7

Project Description:

The statistical report of this unit is shown below. In addition to preparing material for projects conducted in the laboratory of Pathology and Histochemistry, many other investigators received advice and service. These include: Drs. Brodsky, Hesselbach, and Rowe - Laboratory of Infectious Diseases; Drs. Dmitri and Reardon - Laboratory of Tropical Diseases; Drs. Freund, Mallucci, and Tobie - Laboratory of Immunology. Tissues from a small number of animals were prepared for eight other investigators.

Polio vaccine studies, control and experimental aspects continued; tissue from 2,152 monkeys were processed for histopathologic study.

* In addition one technician furnished by DBS for polio vaccine work, approximately one-half a man year.

Serial No. NIAMD-25
1. Pathology & Histochemistry
2. Hematology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Regulation of hemopoiesis.

Principal Investigators: Frederick Stohlman, Jr., and George Brecher.

Other Investigator: Archie A. MacKinney, Jr.

Cooperating Units: Medical Department, Brookhaven National Laboratory.

Man Years
Total: 5.8
Professional: 1.2
Other 4.6

Project Description:

Objectives: Study of red cell and white cell turnover with particular emphasis on the determinants of rates of production and the mechanism of action of these regulants. Attention is also devoted to the physiology and clinical identification of disorders of erythropoiesis.

Methods employed: Rates of red cell production are estimated by means of Fe^{59} incorporation, reticulocyte appearance and bone marrow morphology; destruction utilizing Cr^{51}; modification of rates of erythropoiesis is achieved by varying pO_2 concentrations of inspired air, hypertransfusion, blood loss, irradiation and the administration of erythropoietine.

Cellular proliferation in marrow and peripheral blood is being studied by *in vitro* labeling, using tritiated thymidine and autoradiographs.

Major findings:

1. Bone marrow cellularity influences the plasma level of erythropoietine, presumably through utilization.

2. Short term *in vitro* marrow culture technics cannot be adapted for the assay of erythropoietine. Presumably this reflects the fact that these primarily are maturing rather than dividing systems. It also may be attributed to the fact that erythropoietine may act primarily at the stem cell level and there are inadequate numbers of these cells available to permit detection of an effect.

3. Evidence has been advanced to support the concept that "population pressure" is not of importance in the early release of red cells under maximum stimulation.

4. A shortened stem cell to emergence time has been demonstrated following the administration of erythropoietine in animals in which erythropoiesis was suppressed by hypertransfusion, increased pO_2, and irradiation. Further shortening has been observed under continued erythropoietine administration, suggesting that the action of erythropoietine is not confined to stimulation of early precursor cells.

5. In studies on cellular proliferation it has been demonstrated that an increase in red cell production is achieved through an increase in the number of dividing progenitor cells together with a decrease in the number of normoblasts normally lost by attrition in the marrow.

6. Studies to date indicate that the second regulant of erythropoiesis which we have postulated, is related to the number of circulating red cells. However attempts to document that a postulated feedback from the death of senescent cells is the sole factor has not been possible. In fact bone marrow suppression has been induced by young red cells in the presence of normal oxygen delivery.

7. In vitro studies on thymidine indicate that at the time of withdrawal from the body a certain proportion of cells are in DNA synthesis and continue to synthesize DNA up to 4 hours after withdrawal. However these cells do not enter mitosis nor do other cells enter DNA synthesis after removal from the body.

Significance to the program of the Institute: Anemia is a common complication of arthritis and in many instances may be considered to be a metabolic disorder. Understanding of the mechanisms of red cell regulation are not only of basic interest but should eventually result in improved therapy.

Proposed course of project: Studies on the site of production of erythropoietine and its mechanism of action. Also further studies directed at elucidating the nature of the second regulant of erythropoiesis, the latter to be pursued in both experimental and clinical studies. Patients with anemia of rheumatoid arthritis, polycythemia vera and thalassemia will be the subjects for the clinical investigation.

Cooperative work with Chemotherapy Service, General Medicine Branch, NCI, is being conducted to explore the determinants responsible for thymidine incorporation into DNA *in vitro* and the effect of chemotherapy agents thereon.

Part B included

Annual Progress Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Stohlman, F. Jr.: Observations on the physiology of erythropoietins and its role in the regulation of red cell production. Annals of the New York Academy of Science. 77: 710-724, 1959.

Brecher, G. and Stohlman, F. Jr.: Humoral factors in erythropoiesis. In "Progress in Hematology", 2nd vol., (Tocantins, L. M., ed.), New York and London, Grune and Stratton, 1959, pp. 110-132.

Stohlman, F. Jr. and Brecher, G.: Humoral regulation of erythropoiesis. V. Relationship of bone marrow activity to plasma erythropoietine level. Proc. Soc. Exp. Biol. & Med. 100: 40-43, 1959.

Stohlman, F. Jr. and Brecher, G.: Effect of bone marrow activity on erythropoietine utilization. In "Proceedings of the 7th Congress of International Society of Hematology", Rome, Italy, Il Pensiero Scientifico, 1959.

Thomas, E. D., Lochte, H. L. Jr. and Stohlman, F. Jr.: Attempts to develop an in vitro system for the assay of erythropoietin. J. Lab. & Clin. Med. In press.

Stohlman, F. Jr.: Erythropoietine. Pediatrics 23: 935-936, 1959.

Stohlman, F. Jr.: Preface. In "The Kinetics of Cellular Proliferation" (Stohlman, F. Jr., ed.), New York, Grune and Stratton, 1959.

Stohlman, F. Jr.: Observations on the kinetics of red cell proliferation. In "Kinetics of Cellular Proliferation" (Stohlman, F. Jr., ed.), New York, Grune and Stratton, 1959, pp. 318-324.

Stohlman, F. Jr., editor "The Kinetics of Cellular Proliferation", New York, Grune and Stratton, 1959.

Schmid, R., Brecher, G. and Clemens, T.: Familial hemolytic anemia with erythrocyte inclusion bodies and a defect in pigment metabolism. Blood 14: 991-1007, 1959.

Brecher, G., von Foerster, H. and Cronkite, E. P.: Produktion, Ausreifung und Lebensdauer der Leukocyten. In "Physiologie und Physiopathologie der Weissen Blutzellen (Braunsteiner, H., ed.), Stuttgart, Germany, Georg Thieme Verlag, 1959, pp. 188-214.

Part B:

Brecher, G., Smith, W. W. and Cronkite, E. P.: Strahlenschutz durch Granulozyten. In "Physiologie und Physiopathologie der Weissen Blutzellen (Braunsteiner, H., ed.), Stuttgart, Germany, Georg Thieme Verlag, 1959, pp. 215-226.

Bond, V. P., Fliedner, T. M., Cronkite, E. P., Rubini, J. R., Brecher, G. and Schork, P. K.: Proliferative potentials of bone marrow and blood cells studied by in vitro uptake of H^3-thymidine. Acta Haemat. 21: 1959.

Cronkite, E. P., Fliedner, T. M., Bond, V. P., Rubini, J. R., Brecher, G. and Quastler, H.: Dynamics of hemopoietic proliferation in man and mice studied by H^3-thymidine incorporation into DNA. Annals of the New York Academy of Sciences. 77: 803-820, 1959.

Serial No. NIAMD - 36
1. Pathology & Histochemistry
2. Hematology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Pathogenesis of Experimental Arthritis and Pathology of Rheumatism.

Principal Investigator: Leon Sokoloff, M. D.

Cooperating Units: NCI, Dr. George E. Jay, Jr.

Man Years
Total: 3.2
Professional: 1
Other: 2.2

Project Description:

Objectives: Investigation of factors influencing development of degenerative joint disease in small laboratory animals.

Methods employed: The role of genetic factors in osteoarthritis is being studied by anatomical F1 and F2 hybrids, backcrossed and reciprocals of certain mice are being made.

Major findings: In addition to papers listed below, 6 manuscripts covering the work of the last report, have been or shortly are to be submitted for publication.

In the genetics studies, upwards of 2500 mice have been pedigreed and are at present 8 - 13 months old, to be harvested at 15 months.

A previously undescribed pelvic inflammatory disease has been recognized in males of one strain. It apparently is secondary to occlusion of the urethra by a proteinaceous plug presumed to be of seminal origin. Genetic factors apparently affect it, some crosses being more susceptible than others. The lesion is of interest because it is lethal to the arthritis-prone strain; because it has interfered with biochemical analysis of urine in mice; and because it may prove to be a deleterious effect of celibacy.

Proposed course of the project: The genetics experiments will require a year for completion. The genesis of the g.u. disease will be studied further by castration, breeding, cast examination for structural anomalies, search for a neural cause, possibly related to a gastro-hematopoietic defect.

Part B included Yes

Serial No. NIAMD - 86

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Lerner, E. M. II and Sokoloff, L.: The pathogenesis of bone and joint infection produced in rats by Streptobacillus moniliformis. A.M.A. Arch. Path. 67: 364-372, 1959.

Sokoloff, L., Lillie, R. D. and Anderson, F. O.: A papain digestion apparatus. A.M.A. Arch. Path. In press.

Sokoloff, L.: The comparative pathology of arthritis. In "Advances in Veterinary Science" (Brandly, C. A. and Jungherr, E. L., eds.) Academic Press, N. Y. In press.

Sokoloff, L.: Osteoarthritis in laboratory animals. Lab. Invest. In press.

Sokoloff, L.: Current Comment: In praise of folly. Arth. Rheum. In press.

Serial No. NIAMD - 87
1. Pathology & Histochemistry
2. Hematology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1953

Part I.

Project Title: Studies on normal and abnormal hemoglobins.

Principal Investigator: H. A. Itano

Other Investigators: S. J. Singer
E. Robinson

Cooperating Units: Department of Chemistry, Yale University (Singer)

Man Years
Total: 2.2
Professional: 1.5
Other: 0.7

Project Description:

Objectives: To study the physical chemistry, biochemical genetics, and clinical significance of the normal and abnormal hemoglobins.

Methods employed: Moving boundary electrophoresis; spectrophotometry; column chromatography.

Major findings: The human adult CO-hemoglobins dissociate asymmetrically in acid into unlike subunits and recombine when neutralized (1952 report). The subunits are symmetrical pairs of the α- and β-chains of hemoglobin and are designated α_2 and β_2, respectively. Hemoglobins S and C are abnormal in the β-chain and normal in the α-chain, whereas hemoglobin I is normal in the β-chain and abnormal in α-chain. Acid dissociation and recombination of hemoglobin I with either S or C resulted in the formation of normal adult hemoglobin (A) and a hemoglobin composed of two different abnormal chains. Application of the method to other hemoglobins showed that hemoglobins D_2 E_2 and J are abnormal in the β-chain and that hemoglobin Hopkins-2 (Ho-2) is abnormal in the α-chain. Moreover, a doubly abnormal molecule composed of the abnormal β-chain of hemoglobin S and the abnormal α-chain of hemoglobin Ho-2 has been demonstrated in the hemoglobin of an individual doubly heterozygous for the respective genes for these hemoglobins.

Other workers have reported on the basis of familial studies that hemoglobin S and hemoglobin Ho-2 are controlled by different genetic loci. The present results, which show that these hemoglobins are abnormal in different chains, signify that the α- and β-chains of hemoglobin are controlled by different loci.

Significance to the program of the Institute: Various metabolic disorders in man are known to be associated with inherited absence or decrease of a particular enzymatic activity. A possible mechanism for apparent inhibition of activity is the synthesis of a structurally abnormal enzyme under the control of a mutant gene. Since it is rarely possible to obtain enzymes, especially human enzymes, in adequate purity and quantity for chemical and physical characterization, this postulate is difficult to test directly. However, current concepts concerning the genetic control of protein synthesis are equally applicable to enzymes and to proteins not classified as enzymes. The adult hemoglobins of man, which are obtainable in large quantity and in numerous genetically abnormal forms, thus provide an extremely useful system in which experimental findings will provide generally applicable information on protein synthesis and on the effect of mutations on protein structure.

Proposed course of project: Hemoglobin Hopkins-2 will be further investigated both by recombination and by chemical studies. Other abnormal hemoglobins will be tested by the technique of asymmetric recombination. Any forms that promise to yield new information regarding the genetic control of hemoglobin synthesis will be purified and analyzed chemically.

Part II included Yes

Serial No. NIAMD - 87

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Singer, S. J. and Itano, H. A.: On the asymmetrical dissociation of human hemoglobin. Proc. Natl. Acad. Sci. 45: 174-184, 1959.

Itano, H. A. and Robinson, E.: Formation of normal and doubly abnormal haemoglobins by recombination of haemoglobin I with S and C. Nature 183: 1799-1800, 1959.

Itano, H. A.: Molecular disease. Symposium entitled "Enzymes in Health and Disease", edited by D. M. Greenberg. In press.

Itano, H. A., Singer, S. J. and Robinson, E.: Chemical and genetical units of the hemoglobin molecule. Ciba Found. Symp. Human Biochemical Genetics (Churchill, London, 1959. In press).

Itano, H. A. and Robinson, E.: Properties and inheritance of haemoglobin by asymmetric recombination. Nature. In press. 1959.

Serial No. NIAMD - 38
1. Pathology & Histochemist
2. Hematology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Sulfhydryl (Mercapto) Groups of Hemoglobin Studies on the Nature of the Mercapto-Mercapto Interaction.

Comparison of Normal Adult Human Hemoglobin with Hemoglobin I by 'Fingerprinting'.

The Combining Power of Normal Human Hemoglobin for Nitrosobenzene.

Principal Investigator: Makio Murayama

Cooperating Units: Medical Research Council Unit for Molecular Biology, Cavendish Laboratory, Cambridge, England.

Man Years
Total: 1
Professional: 1
Other:

Project Description:

Objectives: To study the function of SH groups of hemoglobin in relationship to the process of oxygenation.

Methods employed: SH groups of hemoglobins were studied by means of heavy metal ion binding using the rotating platinum wire electrode as an indicator electrode. The data were analyzed mathematically and therefrom the SH-SH interaction constants were derived.

Major findings: Hemoglobin SH groups interact analogous to the well known heme-heme interactions. There are two interaction constants; there is the "toe" and the "shoulder" sigmoid coefficients, respectively, of the binding curve. This finding suggests schizophrenic character of hemoglobin molecule. The underlying mechanism of the mercapto-mercapto (SH-SH) interactions seems to be due to the steric hindrance, as in the heme-heme interactions.

Preliminary studies indicate that the nitrosobenzen "wedge" decreases the energy barrier due to steric hindrance with respect to SH groups; the "wedge" also influences the SH-SH interaction constants. Dr. Max Perutz of Cavendish Laboratory found by the x-ray diffraction studies that nitrosobenzene acted to "open up" the mercury binding sites (SH groups) of hemoglobin molecule.

The combining power of normal human hemoglobin for nitrosobenzene was studied; results indicate that the hemoglobin binds nitrosobenzene about 6 - 10 times more strongly than oxygen. ΔH of the reaction is about -24 kilocalories per mole in contrast to about -10 kilocalories for the process of oxygenation.

A specific chemical difference between the normal adult human hemoglobin and an abnormal hemoglobin I was studied by a method known as "finger printing". Tryptic digest of each protein was subjected to electrophoresis on filter paper and then followed by chromatography to separate the peptides. The result showed that peptide 2S of hemoglobin I contains tryptophan whereas the corresponding one in the normal does not.

There must be other amino acid involved in this genetic change; tryptophan is electrically neutral. Accordingly, the study is in progress to find out the other amino acid which is responsible for the electrophoretic mobility difference of hemoglobin I.

Proposed course of project: Sulfhydryl groups of hemoglobins will be studied by means of heavy metal ion binding using the platinum wire electrode as an indicator electrode. The data thus obtained will be analysed to find out to what extent the sulfhydryl groups interact. It seems that the sulfhydryl-sulfhydryl interaction is extremely important for the understanding of heme-heme interaction accompanying oxygenation.

It is also proposed that a specific chemical difference between the normal adult human hemoglobin and an abnormal hemoglobin I will be studied. This investigation will be carried out by a method known as "finger printing" technique: a tryptic digest of the protein will be made; the hydrolysate is then subjected to electrophoresis on filter paper and then followed by chromatography to separate the peptides.

Quantitative assay of amino acids of the peptide is in progress. Spinco-Spakmann apparatus will be used.

Instrumentation: pH Stat: This is an instrument to maintain a constant pH by continuous addition of acid or base during the reaction. The instrument was designed around a Leeds and Northrup pH meter. It was designed so that a Brown servo amplifier "knows" how much acid or base must be added. The instrument is used in the study of hemoglobin peptides.

Automatic Voltage Scanner: A 10-turn potentiometer is made to scan clockwise 3,600° then stops; and then immediately it reverses itself 3,600° then shuts itself off. At the end of these operations the whole of polarographic circuit is also shut off. The current-voltage curve thus obtained makes possible a more convenient and more precise measurement of constants which characterize the curve.

Part B included Yes

Serial No. NIAMD-ac

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Murayama, M. and Ingram, V. M.: Comparison of normal adult human haemoglobin with haemoglobin I by 'fingerprinting'. Nature 183: 1798, 1959.

Murayama, M.: On the nature of the interaction between binding sites of heavy metals (mercapto-mercapto interactions) in normal human hemoglobin. J. Biol. Chem. 234: ....., 1959.

Murayama, M.: The effect of nitrosobenze on the mercapto-mercapto interaction of human hemoglobin. Federation Proc. 18: 498, 1959.

Murayama, M.: The combining power of normal human hemoglobin for nitrosobenzene. J. Biol. Chem. In press.

(Attachment I)

Serial No. NIAMD-89
1. Laboratory of Pharmacology and Toxicology, NIAMD
2. Section on Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Physical chemical and metabolic factors relating the action of physiological and pharmacological agents in excitable cells such as nerve and muscle.

Principal Investigator: Dr. A. M. Shanes

Other Investigators: Drs. C. Paul Bianchi (Visiting Scientist), N. L. Gershfeld, and S. Winegrad (PHS Fellow)

Cooperating Units: None

Man Years (calendar year 1959):
Total: 3 - 1/3
Professional: 3
Other: 1/3

Project Description:

Objectives: The work is continuing along lines ascertaining the role of ion movement in the bioelectrical properties of excitable tissues through studies of the action of metabolic inhibitors, drugs and ions on nerve, skeletal and cardiac muscle; it embraces the following projects:

(a) the elucidation of the role of calcium ions in muscle contraction from the standpoint of its movement through and binding to cellular aspects of striated tissue, and the extent to which drugs will influence these calcium processes

(b) the systematic study of the role of calcium in the contractile process of isolated vertebrate heart muscle as affected by physiological conditions, and

Part B included Yes

(c) the study of the effects of these pharmacological agents on mono-molecular films of simple molecules and of cellular extracts.

Methods: (a) The rate and quantity of (Ca^{45}) entry and exit in frog striated muscle and ion guinea pig atrial appendage are measured in media which are either modified with respect to its ionic content or to which pharmacological agents affecting contraction are added; comparison is made between non-contracting and contracting conditions. The use of a small amount of cocaine (2 mg % - 14 mg %) eliminates spontaneous twitching in frog striated muscle, and the guinea pig atrial appendage at room temperature does not beat spontaneously. Radiocalcium movements in connective tissue are followed as models for calcium binding in the interstitial connective tissue of muscle.

(b) Left atrial appendages from small guinea pig hearts, suspended by specially prepared clips, are attached to a sensitive transducer designed to quantitatively record changes in the contractions of this biological preparation. The movements of radiocalcium are studied on the same preparation in which the contractions are recorded.

(c) Monolayers of stearic acid, myelin extracts, or purified components of excitable cell membranes are spread on Ringer's solutions and the surface pressure-area relation, which is unique for each surface film, is studied as a function of changing conditions in the Ringer's solution substrate, i.e., calcium concentration, pH, and drugs.

(d) The surface pressure-area relations of surface films of drugs on various substrates are studied to determine the steric relations which exist between drugs which have similar chemical structures but have markedly different pharmacological properties.

Major Findings: 1. Effect of caffeine on Ca^{45} movement in frog striated muscle. Caffeine, in concentrations low enough not to cause membrane depolarization, increases Ca^{45} influx and outflux approximately three fold; the increase in outflux develops at a slower rate than the increase in influx. Caffeine increases influx during potassium depolarization but has no effect on the increased influx due to the initial potassium depolarization. Caffeine affects calcium sites in the membrane which are distinct from those affected by membrane depolarization, yet both sites are related to the contractile process.

The increased calcium outflux due to caffeine is only slowly reversible upon removal of caffeine; the increased outflux is also observed in the absence of external calcium and in the presence of EDTA.

Caffeine has no effect on Ca^{45} uptake or exit from Achilles tendon.

2. The interactions of ions and drugs with surface films of stearic acid. A representative series of drugs from the veratrum alkaloids - veratridine, cevadine, veracevine, and veratramine were used to demonstrate that pharmacological activity may be described in terms of physio-chemical properties of the drugs. The excitatory alkaloids - veratridine and cevadine - were shown to orient

horizontally as well as vertically at the air/water interface; veratramine and the local anesthetics orient only horizontally, and veracevine (a relatively inert agent) is not surface active and consequently shows no preferred orientation at the air/water interface. The manner in which these drugs orient determines, in part, the extent to which they interact with monolayers of stearic acid. The excitatory alkaloids penetrate and interact strongly with the monolayer which subsequently leads to unstable mixed films of stearate and the drug. The local anesthetics and veratramine show weak interactions with the stearate monolayer, and veracevine, which is only slightly surface active does not interact with the stearate film.

Significance to NIAMD Research: Our studies in calcium provides a basis for comparing normal and abnormal tissues; especially in striated, smooth, and cardiac muscles, where the contractile process has been impaired by pathological conditions not related to innervation but to the contractile mechanism itself. Normal and abnormal muscle can now be characterized in regards to changes calcium distribution, association constants and turnover rates during activity. Caffeine can be used as a pharmacological agent which has a direct effect on the cell membrane process involved in excitation-contraction coupling.

Alteration in calcium metabolism in the pathological condition, of arthritis, muscular dystrophies, and contractures can now be examined.

The findings to date on surface films indicate that a means of assessing the nature of the physical chemical interaction of drugs and ions with cell membrane constituents in a model system is at hand. These may ultimately lead to a quantitative description of pharmacological agents in terms of easily measured physiochemical properties. The surface film studies also provide a means for studying enzymatic processes which occur in the cell membrane.

Proposed Course of Project: Calcium binding in muscle and tendon of amphibian and mammal will be characterized by alteration of pH, ionic environment, temperature, and as effected by physiological and pharmacological agents; especial attention will be given to determining the sites of binding in the sarcolemma that are related to the contractile process. The regulation of calcium movement as affected by metabolic inhibitors (iodoacetic acid, dinitrophenol, anoxia, etc.) and by inhibitors of active transport such as the cardiac glycosides.

The study of surface films will extend in directions: (a) studies with other drugs and ions, the physiological and pharmacological effects of which in excitable cells are well characterized, to determine how general is the specificity and parallelism of the physico-chemical film interactions. (b) Studies with film composed of molecules of greater complexity, e.g., the individual components of cellular lipids and purified extracts from tissues with membranes exhibiting different electrochemical properties (e.g., chemically excited membranes such as occur at the myoneural junction as contrasted with electrically excitable membranes).

PHS-NIH
Individual Project Report
Calendar Year 1959

PART F: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Shanes, A. M. and Bianchi, C. P.: The Distribution and Kinetics of Release of Radiocalcium in Tendon and Skeletal Muscle. J. Gen. Physiol. 42:1123-1137, 1959.

2. Shanes, A. M. and Berman, M. D.: The Kinetics of Depression of Potassium Outflux by Cocaine in Toad Sciatic Nerve. J. Pharmacol. Exp. Therap. 125:316-322, 1959.

3. Gershfeld, Norman L. and Shanes, A. M.: Antagonism of Veratrine by Calcium Ion in Monolayers of Stearic Acid. Science 129:1427-1428, 1959.

4. Gershfeld, N. L.: The Influence of Structure on Molecular Orientation at the Air/Water Interface. F-A Studies for Veratrum alkaloids. J. Phys. Chem. 1959. In press.

5. Gershfeld, N. L., and Shanes, A. M.: Stabilizer and Labilizer Effects and Antagonism Demonstrable with Monomolecular Films. J. Physiol. 1959. In press.

6. Bianchi, C. P., and Shanes, A. M.: The Effect of the Ionic Milieu on the Emergence of Radiocalcium from Tendon and from Sartorius Muscle. J. Physiol. 1959. In press.

Honors and Awards relating to this project: None

(Attachment 1)

Serial No. MIAM-90
1. Laboratory of Pharmacology and Toxicology, NIAMD
2. Section on Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Mechanism and therapy of shock and of delayed deaths following burns in humans

Principal Investigators: Drs. Nicholas A. Kefalides, R. Carl Millican, Kehl Markley, III, and S. M. Rosenthal

Other Investigators: A group of Peruvian doctors.

Cooperating Units: Hospitals Loayza, del Nino and Dos de Mayo, Lima, Peru

Man Years (calendar year 1959):
Total: 9
Professional: 7
Others: 2

Project Description:

Objectives: Clinical evaluation of plasma, plasma albumin, and large volumes of saline solution.

The basic mechanism, epidemiology, and treatment of Pseudomonas and Staphylococcus septicemias that follow extensive burns in humans.

Causes of dealyed deaths other than infection.

Methods Employed: The clinical study in Lima, Peru involves comparison of plasma, plasma albumin and large volumes of saline in the therapy of burn shock.

Half of the burned patients receive large doses of gamma globulin intramuscularly on admission and during the first 10 days after.

On the appearance of a positive blood culture for Pseudomonas aeruginosa, a specific antiserum is administered. The antiserum was developed in our laboratory (Dr. Millican) and prepared by Lederle Labs.

Part B included Yes

Extensive chemical, immunological and bacteriological studies are carried out to determine effectiveness of therapy.

Major Findings: The effectiveness of oral saline solutions in burn shock has been substantiated in an eight year study. Only one death from shock has occurred in over 90 adults treated with saline alone while the mortality in the plasma group in adults was 12 per cent. However, in children under 3 years of age, some added benefit was shown for plasma plus saline therapy in mortality from shock (9 % versus 35 %). Whether this is a colloid effect or an immunological effect remains to be established.

The administration of gamma globulin prophylactically has had a definite effect in reducing septicemias. While a more extensive study in needed for final conclusions, the reduction has been statistically significant.

The use of Pseudomonas antiserum has also been too limited for final conclusions. However there have been 5 survivals out of about 15 cases. In the past in over 100 cases there have been no survivors following a positive diagnosis, in spite of intensive antibiotic therapy.

Proposed Course of the Project: Continued study of mechanisms and therapy of shock and of delayed deaths following burns.

(Attachment I)

Serial No. NIAMD-90
Page 3

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Markley, K., Bonanegra, M., Bazan, A., Temple, R., Chiapponi, M., Morales, G.: Clinical Evaluation of Saline Solution Therapy in Burn Shock: II. Comparison of Plasma Therapy with Saline Solution Therapy. J. A. M. A. 170:1633-1640, 1959.

Honors and Awards relating to this project: None

(Attachment I)

Serial No. NIAMD-91
1. Laboratory of Pharmacology and Toxicology NIAMD
2. Section on Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Estimation, metabolism and function of spermine, spermidine, and related polyamines

Principal Investigators: S. M. Rosenthal, D. Dubin, and C. W. Tabor

Other Investigators: Dr. H. Tabor

Cooperating Units: None

Man Years (calendar year 1959):
Total: 4
Professional: 2 - 2/3
Others: 1 - 1/3

Project Description:

Objectives: The importance of these polyamines is shown by their wide distribution in viruses, bacteria, plant and animal cells. Studies are conducted to elucidate their metabolism and function, and relation to desease.

Methods: The amines are studied by isotopic labeling and special chromatographic techniques, using bacteria and animals as test objects.

Major Findings: Biosynthesis of spermidine (see report from Section on Biochemical Pharmacology).

Assay of primary and secondary amines by dinitrofluorobenzene derivatives. It was found that the absorption spectrum of primary amines had a peak at 350 mμ, while the peak of secondary amines was at 390 mμ. Using the 350/390 ratio it was possible to differentiate the polyamines

Part B included Yes

from their acetyl derivatives. The method was further refined to make it several times more sensitive than the one in use.

Acetylation of polyamines. Monoacetylputrescine and two isomeric forms of acetylspermidine were isolated from E. coli cells. They were characterized by behavior on ion exchange resins and paper chromatography, by quantitative acetate and amine determinations on hydrolyzed samples, and by specific activity of C^{14}-labeled compounds. E. coli does not normally contain spermine, but when grown in the presence of spermine, both mono- and diacetyl derivatives were isolated from the cells. A similar result was obtained with Salmonella.

A conjugate of spermine and spermidine with glutathione. A high percentage of the glutathione in E. coli cells was found to exist as a conjugate with spermidine. This was characterized by behavior on ion exchange resins and on paper chromatography, by identification of spermidine, cystine, glycine and glutamic acid after hydrolysis, and by labeling with C^{14} spermidine and S^{35} added to the medium. That it is a true compound was shown by absence of exchange when mixed with C^{14} spermidine. Spermine is not normally present in E. coli, but when added to the growth medium, a similar conjugate with spermine was demonstrated in the cells.

Proposed Course: Further studies of the metabolism, function, and relation to disease of the polyamines.

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Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Dubin, D. T.: The Assay and Characterization of Amines Using 2,4-Dinitrofluorobenzene. J. Biol. Chem. 1959. In press.

2. Dubin, D. T.: Evidence for Conjugates Between Polyamines and Glutathione in E. coli. Biochemical and Biophysical Research Communications, 1959. In press.

3. Dubin, D. T., and Rosenthal, S. M.: The Acetylation of polyamines in E. coli. J. Biol. Chem. 1959. In press.

Honors and Awards relating to this project: None

(Attachment I)

Serial No. NIAMD-92
1. Laboratory of Pharmacology and Toxicology, NIAMD
2. Section on Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Mechanism and therapy of delayed deaths following experimental trauma

Principal Investigators: Drs. R. Carl Millican, Nicholas A. Kefalides, and S. M. Rosenthal

Other Investigators: John D. Rust and Robert C. Jansky

Cooperating Units: None

Man Years (calendar year 1959):
Total: 2 - 1/3
Professional: 1 - 1/3
Others: 1

Project Description:

Objectives: To obtain more potent antiserum by immunization of animals with Pseudomonas aeruginosa for treatment of experimental and clinical P. aeruginosa infections.

To study factors in the cause of delayed deaths in mice following burn injury.

Evaluation of gamma globulin and antibiotics in experimental infections.

Methods Employed: The production of fatal infections with organisms of low virulence in animals made susceptible by burn or tourniquet trauma or by injecting organisms in mucin.

Assay of protective titers of various sera against these infections.

Observe the effects of antibiotic and other chemotherapy reducing the delayed mortality of burned mice surviving the acute shock period.

Part B included No

Major Findings: Rabbit antiserum against Pseudomonas aeruginosa was 800 times more effective than human gamma globulin against fatal mouse infection and was effective even when given 12 hours after infection. Antiserum protected against 8 strains isolated from patients with clinical Pseudomonas aeruginosa septicemias. Purification of the gamma globulin component of antiserum by either DEAE-cellulose chromatography or ammonium sulfate fractionation resulted in a four-fold increase in potency over that of the crude antiserum. In collaboration with Lederle Labs small quantity of refined antiserum was prepared for clinical trial in clinical septicemias in burned patients in Lima, Peru.

Several chemotherapeutic agents have been demonstrated to have a significant effect in reducing the delayed mortality of burned mice surviving the acute shock period. The most effective of these agents was chloramphenicol. Less effective agents were human gamma globulin and the serum of mice convalescing from burn injury, 3 to 8 weeks after injury. No difference was noted in the effectiveness of human gamma globulin and convalescent mouse serum. The combined effect of chloramphenicol and convalescent serum therapy was additive when compared with each therapy alone. A variety of other antibiotics (polymyxin, oleandomycin, chlortetracycline, oxytetracycline, tetracycline, and aigamycin) were ineffective in lowering the delayed mortality after burn.

Proposed Course of the Project: Evaluation of antiserum in the treatment of Pseudomonas septicemias.

Investigation of antibiotics in delayed deaths following burns in mice.

Search for causes of death other than infection in these mice.

(Attachment I)

Serial No. NIAMD-93
1. Laboratory of Pharmacology and Toxicology, NIAMD
2. Section on Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The Biosynthesis of Cholesterol

Principal Investigator: Dr. Kehl Markley, III

Other investigators: Mrs. Elizabeth Smallman

Cooperating Units: None

Man Years (calender year 1959):
Total: 1-1/2
Professional: 1
Other: 1/2

Project Description:

Objectives: (1) To delineate the steps by which mevalonic acid (MVA) is converted to squalene and cholesterol by mammalian tissues. (2) To study the stoichiometry of each step, beginning with the first phosphorylation step of MVA.

Methods: Substrates: D,L 1-C^{14} MVA and D,L 2-C^{14} MVA. Bioassay of enzymatic activity: After inactivation of enzyme by heating, the reaction mixture is quantitatively transfered to Whatman #1 filter paper and chromatographed with n-butanol-HCOOH-H_2O until front has moved 15 cm. Paper then cut into 6 squares, 2.1 x 2.1 cm each, and activity measured in each square. Square #1 contains the product containing the MVA-residue. Enzyme purification: An acetone extract powder of rabbit liver is treated with ammonium sulfate, protamine sulfate, and DEAE-cellulose chromatography. Identification of product: The product, phosphomevalonic acid (P-MVA), separated from pyrophosphomevalonic acid (PP-MVA) by chromatography in t-butanol-HCOOH-H_2O solvent system. P-MVA separated from P_i by chromatography in methanol-NH_3-H_2O). Phosphate determinations by method of Chen.

Major Findings: Mevalonic kinase has been purified 100 fold from an acetone powder of rabbit liver. This enzyme catalyzes the formation of phosphomevalonic acid and ADP from the biologically active isomer of mevalonic acid and ATP. The enzyme was free of ATP-ase and myokinase. Cysteine (or to a lesser extent other SH-compounds), phosphate, and Mg^{++} or Mn^{++} are required for

Part B included ---Yes

activity. Guanosine, uridine, N inosine triphosphates cannot replace effectively adenosinetriphosphate. The enzyme is inhibited by 0.1 umoles of p-chloromercuribenzoate.

Proposed Course: (1) The conversion of P-MVA to the next product will be studied by enzyme purification from rabbit liver. (2) To study the fate of MVA in Lactobacillus acidophilus.

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Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Markley, K., Boconegra, M., Bacon, A., Temple, R., Chiappori, M., Morales, G., and Carrion, A.: Clinical Evaluation of Saline Solution Therapy in Burn Shock. II. Comparison of Plasma Therapy with Saline Solution Therapy. J. A. M. A. 170:1633, 1959.

Honors and Awards relating to this project: None

(Attachment I)

Serial No. NIAMD-94
1. Laboratory of Pharmacology and Toxicology, NIAMD
2. Section on Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The chemotherapy of mouse leprosy

Principal Investigator: Dr. Y. T. Chang

Other Investigators: None

Cooperating Units: The American Leprosy Foundation, Leonard Wood Memorial

Man Years (calendar year 1959):
Total: 1-1/3
Professional: 1
Others: 1/3

Project Description:

Objectives: The evaluation of therapeutic effectiveness of drugs in mouse leprosy. The tissue culture of intracellular parasites.

Methods Employed: Intraperitoneal infection of mice with M. lepræ murium. Tissue culture of macrophages in Leighton tubes.

Major Findings: Dr. Barry's rimino compound, B663, a derivative of phenazine compounds, showed marked suppressive activity in mouse leprosy, approaching the activity of isoniazid, weight for weight; diethyldithiol-isophthalate, marked activity in large doses; N, N'-bis(dl-alpha-pheusthyl)-decamethylenediamine (SU 4592), N-(p-dimethylaminocinnamyl)dodecylamine (SU 5044), only weak activity; phosphanilic acid, bis(p-aminophenyl)phosphinic acid, bis(dimethylaminophenyl)phosphinous acid, 3-methoxy-4-amino-4'-acetylamino-diphenyl sufone, a derivative of 9, 12-diketo-10-octadecenoic acid (ERL 244), levo-3-methoxy-10-(3'-dimethylamino-2'-methyl-1'-propyl)-phenothiazine (Neozinon), and 5-heptyl-2-thiohydantoin, no activity.

The median survival time (ST50) of normal mice was 576 days, and that of untreated leprosy control, 121 days. The ST50 of animals treated with various drugs were as follows: DDS, 168 days; streptomycin, 187 days; nicotinamide, 228 days; pyrazinamide, 256 days; isoniazid, 312 days; and kanamycin, 460 days.

Part B Included Yes

Marked improvement in the cultivation of M. leprae murium in tissue culture of macrophages was obtained by using a medium contained unfilbered horse serum, Hanks' balanced salt solution, beef embryo extract and freshly prepared spleen homogenates from young mice. In one experiment, the macrophages were maintained in good condition for 73 days, although the cell population decreased to about one third. At the end of the experiment, the total number of bacilli increased 6.5 times, and the average length of bacilli increased 2.6 times. Therefore, the bacillary mass increased a total of 16.5 times with only about one third of the macrophages left. Had the other two thirds of macrophages the same chance to develop, there would be much higher increase of the bacillary mass.

Proposed Course of Project: The leprosy studies are carried out in ecoperation with the American Leprosy Foundation (Dr. Cheng in on a Fellowship from them). The results are applied to their clinical evalnation studies.

Continuation of evaluation of drugs in mouse leprosy, using both long-term and short-term techniques. Continuation in the study of tissue culture of M. leprae murium and M. tuberculosis.

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Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Chang, Y. T.: Effects of Kanamycin, Streptoverticin, Paramomycin, Novobiocin, and Ristocetin on Murine Leprosy. Amer. Rev. Tuber. Pulm. Dis. 78:673, 1959.

2. Chang, Y. T.: Evolution of Murine Leprosy. Amer. Rev. Tuber. Pulm. Dis. 79:805, 1959.

3. Chang, Y. T.: More About the Phenazine Dyes Antituberculosis Activity in the Phenazine Series. Leprosy Briefs 10:37, 1959.

4. Chang, Y. T. and Doull, J. A.: Mercaptan Compounds in Tuberculosis and Leprosy. Leprosy Briefs 10:41, 1959.

Honors and Awards relating to this project: None

(Attachment I)

Serial No. NIAMD-95
1. Laboratory of Pharmacology and Toxicology, NIAMD
2. Section on Pharmacology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Toxicologic studies of iodates

Principal Investigator: Dr. S. H. Webster

Other Investigator: Mr. E. F. Stohlman

Cooperating Units: Dr. Benjamin Highman, NIAMD Pathology S1

Man Years (calendar year 1959):
Total: 2 - 2/3
Professional: 2 - 1/3
Others: 1/3

Project Description:

Objectives: The toxicology of iodates as a basis for use in iodate salt.

Methods Employed: Investigation of analytical methods for determination of iodate and iodide in biological materials; study of distribution and excretion of KIO_3 after various routes of administration to rodents.

Major Findings: Sensitive microchemical methods for identifying iodate and iodide ions in urine, without the use of ashing, have been devised. The tests used depend upon the liberation of iodine which is subsequently identified by means of the sensitive starch-iodine reaction. Certain organic substances, which interfere in these tests, were present in nearly all urines examined. One group of substances, which includes resorcinol, phenols, and ascorbic acid, is capable of uniting or binding iodine; the other group, which includes thiocyanate, ascorbic acid, and methionine, is capable of rapidly reducing iodate in acid solution. Nearly all of these interfering substances can be eliminated by a single treatment with activated charcoal. Minimal detectable amounts of KIO_3 and KI have been found to be 5-17.5γ KIO_3 or 150-175γ KI per ml. of urine.

A modification of the above method, to permit quantitative evaluation of iodate in urine, has been developed and is undergoing tests for sensitivity and reliability. The most difficult aspect of this matter is the selection of a stable standard for this labile material.

Part B included Yes

Proposed Course of Project: (1) Testing of a quantitative method for the determination of iodate in urine and biological materials.

(2) Study of the distribution and excretion of KIO_3 after various routes of administration to rodents, using improved analytical techniques.

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Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Webster, S. H., Rice, M. E., Highman, B., and Stohlman, E. F.: The Toxicology of Potassium and Sodium Iodates. II. Subacute Toxicity of Potassium Iodate in Mice and Guinea Pigs. Toxicology and Applied Pharmacology, 1:87-96, 1959.

Honors and Awards relating to this project:

Since February 1959 I have performed the duties of Chairman of the NIAMD Editorial Board. This has involved the handling of 300 manuscripts, ranging from abstracts to chapters of books. Each manuscript required reading by one or more referees. When no Board member was qualified the Chairman sought and received assistance from members of other Institutes or occasionally from specialists outside the NIH. Chairman evaluated each reviewer's comments, particularly when the manuscript was not recommended for publication or was severely criticized. A few manuscripts required revision before final approval was granted. The files, which had not been revised since 1951, were completely examined and brought up to date. A new card index was set up by subject and authors and a card file system was started. This makes it possible to instantly ascertain the status of any manuscript being processed. About one-quarter of the Chairman's time is occupied with these duties.

(Attachment I)

Serial No. NIAMD-96
1. Laboratory of Pharmacology and Toxicology, NIAMD
2. Section on Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Fatty changes in mice induced by short-time fasting

Principal Investigator: Dr. S. H. Webster

Other Investigator: Mr. E. F. Stohlman

Cooperating Units: Dr. Benjamin Highman, NIAMD Pathology 81

Man Years (calendar year 1959):
Total: 2-2/3
Professional: 2-1/3
Others: 1/3

Project Description:

Objectives: Determination of the duration of fasting necessary to produce observable fatty changes in such organs as liver, heart, kidneys and adrenals of mice and the time required for reversal of such changes.

Method Employed: Determination of total fat content and gross and microscopic examination of above organs before, during and after fasting. Also, study of organ weight and body weight changes corresponding to these three periods.

Major Findings: Fasting mice for 7 hours was found to produce no demonstrable fatty changes in the liver, kidneys, heart or adrenals. However, by increasing the fasting time to 16 hours, marked fatty infiltration was noted in liver and kidneys and slight changes were seen in heart and adrenals. On refeeding, the heart, adrenals, and kidneys usually had a normal appearance 24 hours later. However, fat in the liver persisted for at least another 24 hours. In addition, the body weight failed to return to its original value within 24 hours.

It is known that the material responding to fat stains in the mouse liver is neutral fat rather than phospholipid or cholesterol. Since direct methods for the estimation of neutral fat were not available, indirect analyses of other lipids were required. This made analysis of organs of individual mice very difficult, if not impossible.

Part B included NO

Proposed Course of Project: A new chemical method for the direct determination of neutral fat is now being developed elsewhere in the NIH and it is expected that this can be adapted to use in the present study.

The effect of fasting on mice for periods between 7 and 16 hours will be studied in more detail. Additional data will be secured on the change of organ weight and body weight taking place during fasting and refeeding and this will be correlated with the histologic changes taking place during these intervals. Such information should be of great value in oral toxicity studies involving fasting mice since it is often uncertain at autopsy whether fatty changes are caused by voluntary fasting, involuntary fasting, or by action of an administered drug.

Serial No. NIAMD-____97
1. Pharmacology & Toxicology
2. Biochemical Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Metabolism of Histidine, Histamine and Related Imidazoles

Principal Investigator: Herbert Tabor

Other Investigators: Gavin Crowley, John Wolff, Alan Peterkofsky, Hugo Bauer, Virginia Childs

Man Years (calendar year 1959):
Total: 4-1/2
Professional: 3-1/2
Other: 1

Project Description:

Objectives: To study the biosynthesis, intermediary metabolism, and pharmacological activity of these compounds in order to understand better their physiological and pathological role.

Major Findings: Further studies have been carried out on the following enzymatic reactions involved in the metabolism of histidine.

(1) Histidine → urocanic acid + NH_3

(2) Urocanic acid → formiminoglutamic acid

(3) Formimino-L-glutamic acid + tetrahydrofolic acid → 5-formiminotetrahydrofolic acid + L-glutamic acid

(4) 5-Formiminotetrahydrofolic acid → 5,10-methenyltetrahydrofolic acid + NH_3

(5) 5,10-Methenyltetrahydrofolic acid → 10-formyltetrahydrofolic acid

Part B included [X] Yes [ ] No

(1) Further study of reaction 1 in histidine-adapted Pseudomonas has recently been begun. The purification procedure has been revised by the inclusion of a DEAE-column step. Studies in progress (Dr. Peterkofsky) are particularly concerned with further purification, elucidation of the cofactor requirements, and mechanism of the reaction.

(2) Enzyme 2 is being purified from hog liver, using bentonite, DEAE, and calcium phosphate steps. The enzyme has been purified about 100-fold.

(3,4) These two enzymes have been purified about 700-1000 fold, and separated from each other. Formiminotetrahydrofolic acid has been isolated, and characterized. The kinetics and requirements of the two enzymes have been investigated.

(5) The kinetics of this reversible step have been studied; both non-enzymatic and enzymatic factors have been studied. Particularly noteworthy is the rapid hydrolysis rate in the presence of phosphate.

The reversibility of this step at neutral pH is of particular importance since 5,10-methenyltetrahydrofolic acid appears to be the substrate of the reductase, which results in the hydroxymethyltetrahydrofolic acid. This is the pathway involved in serine and methionine biosynthesis.

An enzyme has also been purified from rabbit liver (Dr. Crowley) which carries out the following reaction:

(6) Imidazoleacetic acid + 1-pyrophosphoryl-5-phosphorylribose $\xrightarrow{ATP}$ imidazoleacetic acid ribotide.

This reaction is of particular interest since it is the first *in vitro* demonstration of the possible mechanism for the excretion of imidazoleacetic acid riboside after administration *in vivo* of histamine or imidazoleacetic acid. Dr. H. Remer has synthesized chemically the riboside of both histamine and of imidazoleacetic acid.

Procedures have also been developed (Dr. Wolff) for the analytical separation of ergothioneine, hercynine, and thiohistidine preparatory to studies of their biosynthesis.

Significance to NIAMD Research: Histidine is an essential amino acid, and its products and derivatives enter into many important metabolic relationships. The C-2 of the imidazole ring enters

into the "one carbon" pool, and thus these studies are closely related to other studies on the role of folic acid and vitamin B-12 carried out in this laboratory and elsewhere in NIAMD.

Imidazoleacetic acid riboside is of significance in that it represents a new kind of natural riboside. It has also been used elsewhere as a useful tool for in vivo trapping agent for ribose.

Proposed Course of Project: Further purification and studies of the detailed enzymatic mechanisms involved in the reactions listed, particularly 1, 2, 5, as well as on other imidazoles of biological significance. Where possible, the respective enzymes will be used as tools for the study of problems related to enzyme induction, cell permeability, and mechanism of drug action and drug resistance.

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Part B. Publications

Publications other than abstracts from this project:

(1) Tabor, H., and Wyngarden, L.: Enzymatic formation of formiminotetrahydrofolic acid, 5,10-methenyltetrahydrofolic acid, and 10-formyltetrahydrofolic acid in the metabolism of formiminoglutamic acid. J. Biol. Chem. 234: 1830-1846, 1959.

Serial No. NIAMD- 94
1. Pharmacology & Toxicology
2. Biochemical Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Metabolism of Sialic Acids

Principal Investigator: Leonard Warren

Other Investigators: Cecilia Spearing (M.A. Graduate Student)
John Goldeberry (Summer Employee)

Cooperating Units: S. H. Wollman, Cancer Physiology Section, Laboratory of Physiology, National Cancer Institute, #NCI-906 (Properties of Transplantable Thyroid Tumors) and #NCI-927 (Sialic Acid in the Thyroid Gland)

Man Years (calendar year 1959):
Total: 2
Professional: 1
Other: 1

Project Description:

Objectives: To study the intermediary metabolism and chemical properties of sialic acids in order to understand its role in physiological and pathological states.

Major Findings:

1. A new "thiobarbituric acid assay" for sialic acid has been developed which is 12 times more sensitive than other methods and is specific enough to measure directly the sialic acid content of tissues. The method is unique in that it measures only free sialic acids.

The method has been adapted for paper chromatography and can detect as little as 5 µgm. of sialic acid. It can also detect 0.5 µgm. of 2-deoxyribose.

2. In cooperation with Dr. S. Spicer of this Institute histochemical methods have been developed for the specific staining of sialic acid-containing proteins. The method depends upon coupling

Part B included [X] Yes [ ] No

the free carboxyl group of sialic acids with the basic dyes Azure A or Alcian blue after the periodic acid Schiff reaction. Staining is eliminated by pretreatment of sections with sialidase which specifically removes sialic acids from mucoproteins. The sialic acid content of sections treated with sialidase is markedly decreased and there is an equivalent increase of sialic acids in the section supernatant fluid.

A. The histochemistry of rodent salivary glands has been studied. Rat salivary mucins differ from those of the mouse in that they are resistant to sialidase.

B. The histochemistry of vaginal tissue of the mouse and rat have been studied. The sialic acid concentration of these tissues is subject to hormonal control and increases 5 to 10-fold during pregnancy.

C. In cooperation with Dr. S. K. Wollman (NCI), Dr. Spicer and I have found that the sialic acid contents of several thyroid cancers are increased. Thyroid cancers stain specifically for sialic acid containing mucoproteins whereas normal thyroids do not. The free sialic acid content of the blood and urine of rats with certain sialic acid rich thyroid cancers, is 3 to 4 times higher than normal.

3. In collaboration with Miss C. Spearing we are purifying neuraminidase (sialidase) from the culture fluid of cholera (1800 x purified) and Clostridium perfringens (40 x purified). She is also isolating N-acetylneuraminic acid from human plasma. These are preliminaries to studies on:

a. The specificities of sialidases from various sources.

b. The mechanism of inhibition of influenza virus hemagglutination by mucoprotein.

4. In collaboration with Dr. B. S. Blumberg of this Institute samples of human serum have been freed of virtually all their bound sialic acid by means of purified neuraminidase. Sets of human sera had been selected on the basis of their differing genetic characteristics as determined by their patterns of binding thyroxine, iron and hemoglobin. These sera have been compared with their corresponding untreated samples by observing migration of bands in an electrophoresis apparatus. We have observed in sialic-less sera:

A. A marked slowing of these bands moving to the positive pole.

B. A regular change of pattern of bands.

C. Changes in, but not abolition of, the ability of certain proteins to bind thyroxine, iron and hemoglobin.

5. A study on sialic acids in fish eggs is now drawing to a close. The finding of large amounts of sialic acids in trout eggs (70 µgm/egg), half of which is free, was reported last year. It has now been found that both N-glycolyl and N-acetyl neuraminic acid are the forms of sialic acid present in the trout egg.

6. Studies on thyroid physiology have been carried out with Drs. S. H. Wollman and R. W. Bates. The administration of propyl thiouracil to rats causes a lowering of the sialic acid concentration of the thyroid gland. However the total amount of sialic acid per gland remains constant since there is a corresponding increase in size of the gland. On the other hand, the sialic acid concentration and total amount of sialic acid in the thyroid gland decreases when T.S.H. is given to chicks, in Dr. Bates' I^{131} depletion assay for T.S.H. The sialic acid concentration in the thyroid follows the I^{131} depletion fairly closely.

Pure bovine thyroglobulin contains 1.2% sialic acids (N-acetyl-neuraminic acid) and since this protein comprises about 70% of the gland protein we feel that the measurement of sialic acid in the thyroid is a chemical estimation of its thyroglobulin content.

7. Studies on human urine. Four carbohydrates substances have been detected in human urine by means of the thiobarbituric acid assay. With the assistance of Mr. John Goldsberry (a summer worker) 60 liters of human urine have been processed through de-ionizing columns and large cellulose columns and certain of these urinary constituents have been purified. Two of the substances are neutral for they do not go on to Dowex-1 or Dowex-50 resins. One of these is apparently a 2-deoxysugar but is not any of the common 2-deoxysugars. There is about 0.25-0.5 mg. % of this sugar in human urine. We have about 40-60 mgm. of this material almost pure. A second neutral sugar is probably a 3-deoxysugar. A third substance is a deoxysugar which is picked up by Dowex-1 formate but can be eluted with 0.1 N formic acid. The fourth material has been found to be sialic acid. There is approximately 3 to 5 mg. % sialic acid in human urine.

8. In conjunction with Dr. R. K. Jakoby, over 160 samples of cerebrospinal fluid samples, both normal and pathological, have been analysed. No striking correlations have been established between the content of free or bound sialic acid and pathological states. The normal levels of free sialic acid is 0.4 mg. % and that of bound sialic acid is 1.3 mg. %. These values are considerably lower than those determined by less specific and sensitive methods. The form of sialic acid bound in cerebrospinal fluid is N-acetylneuraminic acid.

Significance to NIAMD Research: Sialic acid is found in relatively large amounts in many mucopolysaccharides. Little is known of its function. The study of sialic acid and its metabolism is of special relevance to an understanding of rheumatic processes, cystic fibrosis of the pancreas, fertilization, nerve function, bacterial and viral infections, and many other processes.

Proposed Course of Projects: To complete and extend investigations listed.

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Part B: Publications

Publications other than abstracts from this project:

(1) Warren, L.: The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234: 1971-1975, 1959.

(2) Warren, L.: Thiobarbituric acid spray reagent for deoxy sugars and sialic acids. Nature, in press.

(3) Spicer, S., and Warren, L.: Histochemistry of sialic acids containing mucoproteins. J. Cytochem. Histochem., in press.

(4) Warren, L.: Nucleotides and nucleosides. In Crosnberg, D. M. (ed.): Chemical Pathways of Metabolism. New York, Academic Press, 1960. In press.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Enzyme and Endocrine Studies on Tryptophan and Nicotinic Acid Metabolism

Principal Investigator: Alan N. Mehler

Other Investigators: Celia Asnien

Man Years (calendar year 1959):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: To isolate the individual steps in the sequence of reactions resulting in nicotinic acid formation, to study the properties of the enzymes involved, and to describe the intermediate metabolites. With the reactions available, to study the relation of these enzymes to altered metabolic conditions.

Methods Employed: Enzymes are obtained from various sources and purified by the variety of methods currently used in this field. Chemical and physical, especially spectrophotometric, methods are used to measure enzyme activity and to identify products. Possible substrates and products are synthesized by conventional organic chemical techniques. Isotopic compounds are synthesized and radioactivity is measured to follow the course of reactions *in vivo* and *in vitro*. Animals are treated to produce altered metabolic states, and enzymes from such animals are assayed. Methods have been adapted to assay enzymes in tissue culture and organ culture preparations.

Major Findings: The nature of the interaction of endocrine factors on the control of the level of picolinic carboxylase in liver has been explored further in collaboration with Mr. McDaniel. The enzyme level is increased through an effect of cortisone, and this effect is opposed by the combined effects of insulin and growth hormone, but not by either alone. The cortisone effect is also opposed by thyroxin.

Part B included [X] Yes [ ] No

3-Hydroxyanthranilic oxidase, found in normal livers at high levels, could not be detected in several strains of tissue culture cells, presumed to be mouse liver cells. The enzyme was also not found in a solid tumor derived from one of the tissue culture lines, but was found in a mouse hepatoma that had been transplanted subcutaneously for several years. The tissue culture cells and tumors were provided by Dr. Virginia Evans of the National Cancer Institute.

Studies on the chemical reactions of the unstable product of 3-hydroxyanthranilic acid oxidation have added support for the proposed structure: 2-amino-3-carboxy-5-formyl-2,4-trans,cis-pentanoic acid.

Isotope trapping experiments with ring-labeled 3-hydroxyanthranilic acid showed conversion to quinolinic acid but not to picolinic acid in normal rats.

Significance to NIAMD Research: Two lines of inquiry are related to NIAMD research. One is a study of the reactions that influence niacin metabolism in order to gain more insight into the biochemistry of this vitamin. The other is the analysis of the effect of hormones on liver enzymes, which may give information about the nature of the metabolic lesions in diabetes.

Proposed Course of Project: Attempts will be continued to find *in vitro* systems for demonstrating the effects of hormones on the level of picolinic carboxylase. The metabolism of 3-hydroxy-anthranilic acid will be investigated further.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Publications

Publications other than abstracts from this project:

(1) Mahler, A. H.: Metabolism of 3-hydroxy-anthranilic acids in animals. 4th Internat. Cong. Biochem. 13: 164-171, 1959.

Serial No. NIAMD- 100
1. Pharmacology and Toxicology
2. Biochemistry of Amino Acids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Biochemistry of Sulfur-containing Compounds

Principal Investigator: Simon Black

Other Investigators: Miss Blondel Hutson, Dr. Norman Bauman, and Dr. John F. Thompson.

Man Years: (Calendar Year 1959)

Total:	2.8
Professional:	2
Other:	0.8

Project Description:

Objectives: A long term objective is discovery of enzymatic mechanisms involved in the synthesis of constituents of living tissue, particularly of proteins.

Methods: Organisms and extracts of organisms are tested for their ability to convert certain sulfur-containing compounds to new substances, or to form or transform known sulfur compounds. Chemical, chromatographic, radiochemical, and radioautographic methods are used. Mechanisms of formation are elucidated by classical enzymological methods.

Major Findings: (1) An enzyme system isolated from yeast, which catalyses the reduction by TPNH of L-methionine sulfoxide to methionine, has been studied and characterized. It is found to consist of three separable protein fractions, designated I, II and III. In addition to sulfoxide reduction, a non-specific reduction of disulfides is catalysed by the combined action of I and II:

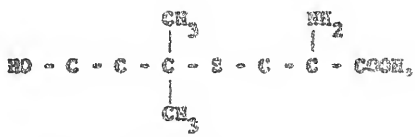
$$CH_3\text{-}\overset{O}{\overset{\|}{S}}\text{-}R + TPNH \xrightarrow[+\ III]{I\ +\ II} CH_3\text{-}S\text{-}R + TPN$$

$$R\text{-}S\text{-}S\text{-}R + TPNH \xrightarrow{I\ +\ II} 2\ RSH + TPN$$

(2) In addition to these enzymes a new non-specific disulfide reductase has been found involving glutathione:

$$2\ GSH + R\text{-}S\text{-}S\text{-}R \longrightarrow GS\text{-}SG + 2\ RSH$$

(3) The biosynthesis of selinine,



has been studied further with indications that it originates in some manner from intermediates of cholesterol formation.

Significance to NIAMD Research: Knowledge of the intimate chemical transformations in living cells will serve as a basis for better understanding of the nature of disease and its more intelligent treatment.

Proposed Course of Project: The discovery of two new enzymatic reductions of disulfides opens possibilities for study of such groups and their interconversions in protein molecules, processes considered to have great importance in physiological transformations.

Part B: Publications

Publications other than abstracts from this project:

(1) Wolff, E. C., and Black, S.: Formation of the methylthiol ester of 3-phosphoglyceric acid catalyzed by glyceraldehyde-3-phosphate dehydrogenase. Arch. Biochem. and Biophys. 80, 236 (1959).

(2) Black, S. Biochemistry of newer sulfur-containing amino acids, in "Amino acid and protein metabolism", published by Ross Laboratories, Columbus, Ohio (1959).

(3) Black, S. β-Aspartyl phosphate and aspartic-β-semialdehyde, Methods in Enzymology, Vol. 6, Article 284, (in press).

(4) Black, S. Conversion of aspartic acid to homoserine, Method in Enzymology, Vol. 6, Article 111, (in press).

(5) Black, S. Glycerate kinase, Methods in Enzymology, Vol. 6, Article 46, (in press).

Serial No. NIAMD.- 101
1. Pharmacology and Toxicology
2. Biochemistry of Amino Acids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Biosynthesis of Gramicidin J

Principal Investigator: Dr. K. Kurahashi

Other Investigators: Mrs. Akie Sugimura

Man Years (calendar year 1959)
Total: 1.9
Professional: 1.5
Other: 0.4

Project Description:

Objectives: To elucidate the mechanism of biosynthesis and intermediary metabolism of a cyclic hexapeptide, Gramicidin J, which is produced by Bacillus brevis.

Methods Employed: The enzymatic incorporation of carbon-14 labeled amino acids into Gramicidin J, and possible intermediary substances in extracts of Bacillus brevis is being studied with the aid of paper chromatography, ion exchange resin chromatography and paper electrophoresis.

Major Findings: C^{14}-labeled L-phenylalanine, together with various energy sources and co-factors, gives rise to an unknown C^{14}-labeled compound which yields C^{14}-phenylalanine upon acid hydrolysis. This compound appears to be rather closely related to Gramicidin J, but not to be identical with it, for the two can be completely separated by chromatographic procedures. That they are closely related is indicated by the fact that strong acid hydrolysis of either one gives rise to phenylalanine, proline, valine, ornithine and leucine.

The incubation mixture for the production of this compound has been simplified. A clear solution of soluble enzymes plus ATP and DPN brings about incorporation of C^{14}-labeled amino acid into the unknown substance. C^{14}-labeled amino acids, L-C^{14}-isoleucine, L-C^{14}-alanine, L-C^{14}-tyrosine, L-C^{14}-leucine, L-C^{14}-threonine, which are not the component amino acids of Gramicidin J, were not incorporated into the unknown product.

By the use of the technique of P^{32}-inorganic pyrophosphate exchange with ATP, it was found that the only D-amino acids activated by the cell free extract of B. brevis is D-phenylalanine which is one the component amino acid of Gramicidin J. D-leucine which is one of the component amino acids of Gramicidin J. was not activated.

Proposed Course of Project: It is planned to pursue further the identification of the unknown compound in relation to Gramicidin J. and to study further the conditions of its formation.

It is also planned to study the effect of fractionation of the crude extract by ammonium sulfate on the incorporation of C^{14}-labeled amino acids into the unknown compound.

Part B:

Publications:

K. Kurahashi and A. Sugimura: Purification of galactose-1-phosphate uridyl transferase from Escherichia coli mutant. (Manuscript submitted to J. Biol. Chem.).

B. M. Kalckar, K. Kurahashi and E. Jordan: Hereditary defects of galactose metabolism in Escherichia coli mutants. Proc. Nat. Acad. Science. (in press).

Serial No. NIAMD-102
1. Pharmacology and Toxicology
2. Biochemistry of Amino Acids
3. Bethesda, Maryland

Project No: 66301-32

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The cytological localization of proteins and enzymes by the fluorescent antibody technique.

Project I - Studies on Streptococcal hyaluronidase and antihyaluronidase.

Principal Investigator: Dr. E. W. Emmart

Other Investigators: William A. Turner

Man Years: - 50% time on hyaluronidase studies; 45% on other research projects, and 5% of time spent with Board of Civil Service Examiners.

Project I Description: The studies on the cytological localization of injected streptococcal hyaluronidase by the Coons fluorescent antibody technique has been extended to include the localization of the enzyme following streptococcal infection. In these studies the invading organism has been tagged in tissue sections with Group C antisera coupled to lissamine rhodamine B 200 while the enzyme hyaluronidase which it elaborates in situ was tagged with rabbit antihyaluronidase globulin coupled to fluorescein.

The paper entitled "Studies on Streptococcal Hyaluronidase and Antihyaluronidase. III. The production and cellular localization of hyaluronidase following streptococcal infection, by E. W. Emmart and W. A. Turner has been approved for publication and submitted to Journal of Histochemistry and Cytochemistry.

Project II - Localization of muscle proteins in the conduction bundle of the beef heart.

Principal Investigator: Dr. E. W. Emmart

Other Investigators: Dr. Einar Halander (Visiting Scientist, Dept. of Anatomy, University of Gothenburg, Sweden).

Dr. H. M. Pullman - N.I.D.R. - L.M.P.

Project II Description: By means of antibodies to myosin, actin and sarcoplasmic proteins conjugated to fluorescein these proteins have been localized in the Purkinje cells of the conduction bundle of the beef heart.

Papers in Progress and Nearing Completion - Distribution of muscle proteins in the node and bundle of the conduction system of the beef heart, by E. W. Emmart and E. Holander.

Published Papers: "Localization of myosin in the conduction bundle of the beef heart". E. Holander and E. W. Emmart, Proc. Soc. Exp. Biol. and Med. 1959, 101, 838-842.

Project III - A Histochemical Study of Enzyme Substrates in the Cells of the Conduction System of the Beef Heart.

Principal Investigator: Dr. E. W. Emmart

Other Investigators: Dr. H. M. Fullmer - N.I.D.R. - L.H.P.

Project IV - Studies on Antibody to Callicrein.

Principal Investigator: Dr. Marion Webster - N.H.I. - Project No. 110

Other Investigators: Dr. E. W. Emmart

Project Description: The hypotensive enzyme callicrein has been isolated from human urine and from pancreatic tissue. Following injection in rabbits antisera has been secured. A study of the inhibitor action on the enzyme of various serum fractions is in progress. If antiglobulin of high titre is obtained after fractionation further studies on cellular absorption of callicrein with fluorescent antiglobulin will be undertaken.

Project V - Cytological localization of insulin.

Principal Investigator: Dr. Glenn A. Mortimore - Clinical Endocrinology Branch (NIAMD)

Other Investigators: Dr. E. W. Emmart - NIAMD - LPT
Dr. Frank Tietz - NIAMD - LBM.

Project Description: A derivative of insulin which retains its hypoglycemic action has been prepared and conjugated to fluorescein isothiocyanate. The absorption of this material in hepatic cells is under observation with the fluorescence microscope.

Part I

Papers published:

"Localization of myosin in the conduction bundle of the beef heart". by Drs. E. Helander and E. W. Emmart, Proc. Soc. Exp. Biol. and Med., 101, 838-842, 1959.

Serial No. NIAMD - 102
1. Pharmacology and Toxicology
2. Biochemistry of Amino Acids
3. Bethesda

FHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Structural Basis of Enzyme Activity.

Principal Investigator: T. Viswanatha

Other Investigators: None

Man Years (calendar year 1959)
Total: 1.4
Professional: 1.0
Other: 0.4

Project Description:

Objective: To determine the relation of the intimate molecular structure of an enzyme to its catalytic activity.

Methods: Trypsinogen is acetylated and then degraded by the action of pepsin to yield an enzymatically active fragment of the original zymogen. This is studied further with a variety of chemical and enzymatic techniques.

Major Findings: An active enzyme fragment of trypsinogen can be further reduced in size by 10 amino acid residues by treatment with leucyl amino peptidase plus Mg^{++} ions. With Mn^{++} ions further degradation is possible leading eventually to loss of activity. Mn^{++} appears to cause the rupture of a particular bond in the molecule exposing a new end group for attack by leucyl amino peptidase.

In collaboration with Drs. W. R. Lawson and B. Witkop it was found that treatment of trypsinogen with N-bromosuccinimide leads to differential destruction of the "specificity determining structure" and the "catalytic site" of the enzyme.

Significance to NIAMD Research: It is hoped that the basic understanding of enzyme function, sought in this work, will increase our knowledge of physiological functions and of disease processes.

Proposed Course of Project: It is expected that this work will continue on its present course until the enzyme structure being studied is fully comprehended.

Part B:

Publications:

1. T. Viswanatha. In the mechanism of enzyme action, Oakridge Symposium on Enzyme Reaction Mechanisms. (April 1959).

2. J. E. Folk, J. A. Gladner and T. Viswanatha. A simplified chromatographic purification of leucyl amino peptidase. Biochim. Biophys. Acta. 36, 256 (1959).

3. T. Viswanatha, W. B. Lawson, B. Witkop. Action of N-Bromosuccinimide on trypsinogen and its derivatives. Biochim. Biophys. Acta. (in press).

Serial No. NIAMD-104
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Effects of hypoxia on physiological and pathological mechanisms in animals.

Principal Investigator: Paul D. Altland

Other Investigators: Edwin G. Thompson, Edna G. Thompson, Milton Parker

Cooperating Units: Dr. Benjamin Highman, Section on Pathologic Anatomy, Laboratory of Pathology and Histochemistry, NIAMD-31

Man Years
Total: 4
Professional: 1
Other: 3

Project Description:

Objectives: (1) To determine the physiologic mechanisms which influence altitude acclimatization and tolerance:
(a) Evaluation of the relation of body temperature and rectal to altitude tolerance in rats.
(b) Study of influence of aortic and mitral insufficiency on survival to acute and chronic exposures to high altitude in dogs.
(c) To determine the influence of exercise on altitude tolerance of rats.
(d) To discover the nature of altitude tolerance in birds.
(e) To study changes in serum enzyme levels induced by altitude exposure in dogs.

(2) To establish the role of hypoxia in the development of disease:
(a) To determine the rate and degree of development of experimental atherosclerosis in rabbits and chickens exposed continuously to altitude.
(b) To determine the influence of hypoxia on the immune response of animals.
(c) Hypoxia renders rats highly susceptible to endocarditis. To study other factors which influence susceptibility to endocarditis.

Methods employed: Altitude exposures conducted in decompression chambers. Physiologic, hematologic, and pathologic techniques used.

Major findings: Rats restrained immediately before rapid ascent to altitude (33,500 ft. at 2,000 ft./min.) die sooner than unrestrained rats. Such reduced tolerance is associated with an increase in the oxygen requirements as a result of struggle to escape restraint. With slow ascent (2-1/2 to 4 hours to reach 33,500 ft.) the tolerance was increased in both restrained and unrestrained rats. The body temperature of the rats dropped to low levels before reaching the critical altitude thus favoring better tolerance. Restraint tends to hasten the fall in body temperature commonly associated with an exposure to altitude thus providing greater tolerance to altitude. Prealtitude induced hypothermia induced by restraint plus exposure to a room temperature of 3 to 5 degrees C. for 2 hours afforded complete protection to exposure to an altitude of 33,500 ft. for 6 hours.

Normal dogs and those with surgically induced aortic and mitral insufficiency survived 4 hour exposures to 30,000 and 32,000 ft., whereas at 34,000 and 36,000 the operated dogs showed a higher mortality. Prolonged intermittent exposure of the operated and unoperated dogs to 30,000 ft. resulted in no difference in tolerance or tissue changes. Of particular significance was the finding of nonlipid arteriosclerotic plaques in the aorta of some of the young dogs exposed to altitude for several weeks. The lesions were more severe in character with increasing numbers of altitude exposures. The lesions are attributed largely to hypoxia.

Endocarditis in dogs with aortic insufficiency was induced by a single injection of Staphylococcus aureus. Penicillin treatment was completely effective if administered within 6 hours after inducing the infection. If treatment was delayed 24 hours symptoms often occurred after cessation of therapy. Proliferative glomerulonephritis, which developed in nearly all dogs given delayed treatment, persisted despite therapy.

Significance to NIAMD research: Results indicate the importance of the body temperature in altitude tolerance of animals and emphasize the important role that restraint has in influencing the body temperature of animals. Findings show that dogs with aortic insufficiency and mitral insufficiency have a surprisingly high altitude tolerance despite the cardiac disease. The occurrence of nonlipid arteriosclerotic plaques in dogs exposed to altitude suggests that hypoxia may play an important role in the etiology of this disease.

Proposed course of project: To conduct experiments to accomplish listed objectives.

Part B included. No.

Univ[illegible] Project [illegible]
Orlando, Year 1959

Part B: Honors, Awards, and Publications.

Publications other than abstracts from this project:

Highman, B., Maling, H. M., and Thompson, D. C.: [illegible] transaminase and alkaline phosphatase levels after [illegible] of norepinephrine and epinephrine in dogs. Am. J. Physiol. 196: 636-640, 1959.

Bartlett, Jr., D. J., and Altland, P. D.: Effect of [illegible] on altitude tolerance in the rat. J. of Applied Physiol. 395-396, 1959.

Bartlett, Jr., D. J., and Altland, P. D.: [illegible] temperature and [illegible] on altitude tolerance in the rat. J. of Applied Physiol. 14: 783-787, 1959.

Altland, P. D., Highman, B., and Brooks, J.: Effects [illegible] on dogs with valvular heart disease. Publ. Arch. Path. 475-486, 1959.

Highman, B., Altland, P. D., and Pierce, J.: Subacute [illegible] endocarditis and pharmacodynamics in dogs. Effects [illegible] with penicillin and chemotherapy. Circulation Research 987-997, 1959.

Altland, P. D., and Highman, B.: Effects of High [illegible] Cholesterol Fat Feeding (Production of Severe [illegible] Atherosclerosis with Modifications). Accepted for [illegible] by A.M.A. Arch. of Path.

Serial No. NIAMD-105
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Invertebrate Physiology

Principal Investigator: John E. Buck

Other Investigators: Leo Levenbook, Margaret L. Kolster, Helen D. Park, Christyne E. Meece, Vincent Hollis

Cooperating Units: None

Man Years (calendar Year 1959):
Total 6
Professional: 4
Other: 2

Project Description:

Objectives: The long range objectives can be defined as the research interests of the four professionals in the unit. Specifically: (1) Basic induction mechanism in origin of reproductive cells from some cells. (2) Mechanism of protein synthesis in insect metamorphosis. (3) Physical factors in respiration. (4) Biological triggers.

Methods Employed: Carbohydrate metabolism has been followed by chromatographic methods of identification and assay of various types of blood sugars, chemical isolation of tissue of glycogen, and respirometry of intact organisms and of tissues. Enzymatic pathways have been followed by use of radioactive tracers. The aerobic-anaerobic transition zone is being studied by measuring oxygen uptake before, during and after exposure to various partial pressures of oxygen. The biophysical aspects of gas transfer involve dimensional study of the respiratory system, respirometry and computation. Electrometric methods were applied to an intensive study of neurogenic initiation of bioluminescence in fireflies.

Major findings: 1. During the past year, 5 cultures of Hydra showing sterility, 20-25 day cycles of gonad differentiation

have been established by Dr. Park. Although the factor or factors responsible for this periodic sexuality is proving very elusive, this and other results definitely exclude CO_2, culture crowding, frequency of feeding, accumulation of material on culture glassware, and accumulation of soluble metabolites as inducing agents.

2. Dr. Levenbook has found high levels of citrate in the blood of 5 species of insect. These measurements, together with similar data in the literature for 3 insects, suggest that high blood citrate is a biochemical peculiarity of insects. Apparently, however, the citrate titer is not an accumulation due to blockage of later stages in the TCA cycle, because Dr. Levenbook has assayed the 10 separate enzymes of the cycle plus citrate cleavage enzyme, isocitritase and malase synthetase, and found all but the last two present. Furthermore, he has shown that citrate, alpha ketoglutarate, malate, fumarate and pyruvate are completely oxidized by the insect's mitochondria in vitro.

3. Dr. Keister has completed a comprehensive study of the relations of O_2 tension and of temperature to respiration of fly larvae and pupae and already has much comparable data on the adult stage. Beside providing needed basic data on all the developmental stages of a single species, the results have shown that (a) larval respiration is never limited by the physical structure or dimensions of the respiratory openings (in contrast to Dr. Park's finding that it may be so limited in the pupal stage), (b) larval respiration shows a sharp plateau between 10 and 15°, (c) decapitation causes little change in the oxygen uptake rates of one day old flies in the range 0-45°.

4. Miss Mecca has completed a study of the effect of cuticle puncture on respiration of a laboratory moth as a preliminary to metabolic studies requiring injection of materials into the pupa. Although the literature reports a very marked stimulation of respiration after comparable injury to pupae of disparate types of moth, no effect was found in Prodenia, a non-diapausing species.

5. Dr. Buck continued his collaborative work at Woods Hole with Dr. James Case of the University of Iowa on the mechanism of bioluminescence in the firefly. The major findings of the summer include (a) the detection in the photogenic tissue itself of action potentials preceding the flash; (b) the fractionation of the overall response latency into two steps, the first of about 50 msec and the second of about 15, the former of which can be by-passed by intense stimulation; (3) the discovery that a variety of agents, including spermine and serotonine, can disrupt the luciferin's regeneration mechanism so that photocytes flash individually and asynchronously.

Significance to NIAMD research: All the work of this unit can be considered as contributing to the basic biology of metabolism. More specifically, the various projects underway impinge on: intermediary metabolism, cell differentiation, biophysics of gas transfer, endogenous rhythms (biological clocks), and biological triggering (biophysics of excitation).

Proposed course of project: Dr. Levenbook plans to investigate insect organic acid metabolism during various stages of development, and to initiate a study of amino acid turnover and protein synthesis using lysine C^{14}. The other investigators expect to carry on in the directions indicated by their progress reports.

Honors: The appearance of the excellent Vol. 12 of the Proceedings of the IVth International Congress of Biochemistry (see bibliography below) provides concrete evidence of the distinction gained by Dr. Levenbook in being asked to organize the symposium on "Biochemistry of Insects" and of his critical job of editing the volume. For his role in the Congress, he was presented with a "Service to Science" citation by the Minister of Education, Republic of Austria. Dr. Buck was appointed to the Editorial Board of the Biological Bulletin, and elected to the following posts: Board of Trustees, Marine Biological Laboratory; Executive Committee, Marine Biological Laboratory; Vice President, Society of General Physiologists; American National Committee of the International Union of Zoological Sciences. Dr. Buck was asked to cooperate as a Visiting Lecturer in an NSF supported program organized by the American Institute of Biological Sciences "to enable undergraduate and graduate students at small liberal art colleges and universities to meet and become acquainted with leading biologists in the various fields of life sciences." He spoke under these auspices at Pennsylvania State, Lehigh, and Drew Universities, and at Moravian College.

Dr. Park was invited to lecture before the Annapolis Secondary Schools Science Seminar.

Dr. Buck organized a 2 1/2 day symposium on Arthropod Physiology for the American Society of Zoologists at the recent Washington A.A.A.S. convention. Abstracts of the 45 papers presented have been published in Amer. Nat. Vol. 102, No. 3 (Nov., 1968).

Part B included Yes

[illegible] Project Report
[illegible] Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Levenbook, L.: Biochemistry of Insects. (Ed) 252 pp. Academic Press.

Friedman, Stanley: Postflight Flight in Phormia [illegible] and its effect on blood [illegible]. J. Insect Physiol. 3. [illegible] (1959).

McDermott, Frank A. and Bath, John B.: The lampyrid [illegible] of Jamaica. Trans. Am. Ent. Soc. 85: 1-112 (1959).

Innevenne, Filadelfo and Levenbook, Leo: Effect of [illegible] occurrence of 5-methyl cytosine and the free amino acid pattern in insect blood. In press in Biochim et Biophysica [illegible]

Friedman, Stanley: The purification and preparation of [illegible] isolated from Phormia regina Meig. In press in Arch. Biochem. Biophys.

NIH-NHI
Radiation Project Report
Calendar Year: 1959

Part A.

Project Title: Mechanism of the circulatory reaction of sensitive species to synthetic macromolecules

Principal Investigator: Louise H. Marshall

Other Investigators: Charles E. Evans

Cooperating Units: None

Man Years (calendar year 1959)
Total: 3
Professional: 1
Other: 2

Project Description:

Objectives: To study vasodepression and edema formation in rats after Escherichia coli endotoxin and in dogs after polyvinylpyrrolidone (PVP).

Methods: Rats have been prepared carrying permanent cuffs in the abdominal aorta from which arterial blood pressure is recorded throughout experiments in unrestrained animals. Intravenous polyvinylpyrrolidone (PVP) rapidly lowers blood pressure, but 2-3 weeks is more usual. Local effects of the glucan applied on skin capillary permeability have been followed by quantity of dye (T-1824) extravasation, bleeding and passage of fluid into the subcutaneous chamber (plasma formation). Using a differential method, we can measure the increased volume of water about pure cells at pseudoplasma of 3%. In experiments concerned with local edema, (the most important known pharmacologic mediator) we have used Evans blue dye (T-1824) and, recently, we used Evans blue dye (T-1824) and fluorescein isothiocyanate (FITC) conjugated to rabbit gamma globulin (RGG). The Bonderman-Lilienfield method for blood glucose was modified by centrifugation prior to its use on blood samples taken during dextran or PVP. We found that EDTA heparin and oxalate solutions (0.2%) prevented coagulation and glycolysis. The glucose determination on the dogs using the Folin-Wu method. The fluorescein-labeled blood glucose with no significant decrease in glucose permeability by keeping the plasma level of the dye at a minimum

Major Findings: 1. In conscious rats it was possible to completely suppress the reaction to intravenous dextran by the proper combination and dosage of anti-histamine with anti-serotonin drugs. This confirms independent results in anesthetized rats from another laboratory.

2. H. pertussis inoculation of rats and mice increases their susceptibility to exogenous histamine and serotonin. We inoculated rats with suspensions of this organism and found their susceptibility to dextran, a histamine-and serotonin-releaser in rats, to be unchanged. At the same time, counts of mast cells decreased in the skin of the paw dorsum, which is one of the "target areas" of dextran sensitivity. This indicates that the dextran reaction does not depend on the presence of these cells.

3. Our major activity has been investigating the relation of insulin to the rat's dextran reaction. Experiments are described in the literature which show both enhanced and mitigated reactivity to dextran after insulin. We have found that route of administration and dosage level of both dextran and insulin determine the differences in effect. Non-hypoglycemic levels of insulin protect rats against both vasodepression and edema characteristically seen after dextran is injected intravenously. After larger doses of insulin, rats are protected against signs of the dextran reaction but are more susceptible to fatal convulsions. The known effect of insulin on cellular permeability to simple carbohydrates led us to expect an enhanced formation of dextran-induced edema after insulin. We have evidence indicating edema formation may be selectively enhanced by insulin, for although rats do not show edema of the extremities, they are thirsty and bleeding is more generalized.

Significance to NIAMD Research: The investigations described constitute basic research in the physiology of cellular permeability. It has been our attempt to keep the cellular reactions in perspective by studying them within the framework of the organism as a whole.

Proposed course of the project: We shall continue to follow what seem to be promising leads to more complete understanding of why individual species react to certain substances. It is important to test the effect of insulin on the reaction of dogs to PVP, which has an structural relation to the dextrose configuration as does dextran. Because certain antiserotonins are effective in mitigating in rats the capillary hyperpermeability occurring after immobilization and thermal burns,

representative drugs will be surveyed for their effect on the dextran reaction in this species.

No publications, honours, or awards.

Part B. included. No

PHS-416
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Pulmonary Ventilation

Principal Investigator: Heinz Specht

Other Investigators: Howard Kaubach, Roy Milner, Arnaud [illegible]

Cooperating Units: None

Man Years (calendar year 1959):
Total: 6
Professional: 2
Other: 4

Project Description: Studies in pulmonary ventilation and [illegible] physiological phenomena subject to atmospheric influences [illegible] abnormal pressure and gas mixture.

Objectives: The [illegible] objective of the overall [illegible] explore new [illegible] regarding breathing behavior with [illegible] to their physiological significance. In order to account [illegible] this, various [illegible] of physiology must be understood [illegible] to control accurately the experimental situation. [illegible] relation of work to breathing and resistance to breathing [illegible] been sought as limiting factors in normal as well as [illegible] conditions of breathing. A secondary objective will be [illegible] study of adaptation to environmental stresses and [illegible] modify such ventilatory responses.

Methods Employed: Techniques are studied for oxygen [illegible] carbon dioxide production, breath velocity, [illegible] measurements of pressure. New devices [illegible] made for new applications to experimental [illegible] The use of [illegible] including [illegible] without the use of metric weighing has been [illegible] of a study of the effect of [illegible]

Major Findings: [illegible] studied on the effects [illegible] tolerance in man [illegible] have shown that [illegible] underestimation of [illegible] temperature and that [illegible] of hypoxia or [illegible] high altitude. The observations [illegible] further examples [illegible] the subtle factors [illegible] physiological [illegible] by modification [illegible]

Major Findings (cont'd):

An analysis of the temporal lag between alveolar pressure and the resultant mouth air flow in respect to gas density, breathing effort and breath frequency has been published and has shown that assumptions disregarding this phenomenon in attempts to measure airway resistance lead to errors due to the fact that the pressure at the alveolus measured by any of several methods is out of phase with mouth air flow. Gross errors may result since uncorrected observed data would lead one to believe that flow without pressure and no flow at finite pressure exists under certain conditions. This study has not only called attention to this phenomenon, but has also demonstrated its dependence on gas density and rate of acceleration of the breath velocity during different breathing patterns.

Current work on breath velocity patterns indicates that high density gas mixtures have a somewhat less marked effect on the pattern than expected from extrapolations from previous work with low density gas mixtures but the method of assessment requires that much more data be collected and analysed before quantitative effects can be given.

In the course of the work mentioned above the opportunity was taken to utilise the various gas mixtures to initiate the devising of a method of body density measurement which obviates the estimation of lung volume yet uses Archimedes principle of weighing in two different density media. The fact that both media are respirable makes this method attractive and the avoidance of submersion makes it potentially very much more useful than previous methods.

Proposed Course of Project: It is planned to undertake research on pulmonary ventilation in dogs in order to provide a background of information similar to that from human subjects but permitting active intervention in normal processes not feasible with subjects on a scale which will provide statistical analysis.

Part. B included. Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Bartlett, Jr., R. G., Brubach, H. F., Trimble, R. C., and Specht, H.: Airway resistance measurement during any breathing pattern in man. J. Applied Physiol. 14: 89-93 (1959).

Bartlett, Jr., R. G., Brubach, H. F., and Specht, H.: Demonstration of ventilatory mass flow during ventilation and apnea in man. J. Applied Physiol. 14: 97-101 (1959).

Bartlett, Jr., R. G., Altland, P. D.: Effect of restraint on altitude tolerance in the rat. J. Applied Physiol. 14: 395-396 (1959).

Bartlett, Jr., R. G., and Young, M. W.: Free Rooming in the Albino Rat and its effect on restraint hypothermia. J. Applied Physiol. 14: 393-394 (1959).

Bartlett, Jr., R. G.: Effects of restraint on oxygen consumption of the cold exposed guinea pig. J. Applied Physiol. 14: 46-48 (1959).

Bartlett, Jr., R. G., and Altland, P. D.: Relation of body temperature and restraint to altitude tolerance in the rat. J. of Applied Physiol. 14: 765-768 (1959).

Serial No. NIAMD- 108
1. Physical Biology
2. Physical Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The relationship between structure and function in biology.

Principal Investigators: Dr. Koloman Laki and Dr. H. A. Saroff

Other Investigators:

Dr. W. J. Bowen	H. L. Martin
Dr. W. R. Carroll	I. Mueller
Dr. D. R. Kominz	J. Standaert
Dr. E. Helander	J. W. Mealy
Dr. F. Irreverre	E. R. Mitchell
Dr. J. A. Gladner	E. N. Smith
Dr. R. B. Simpson	E. F. Wilson
Dr. Y. Levin	G. Rice
Dr. L. C. Stewart	E. Kenton

Cooperating Units:

Dr. J. O. Davis, LKEM-NHI (Serial No. 236)
Dr. John T. Tripp, LBBP-BS (Serial No.)
Prof. Lester J. Reed, Dept. of Chemistry, Univ. of Texas
Dr. J. F. Thompson, U.S. Plant, Soil, and Nutrition Laboratory, ARS U.S. Dept. of Agriculture
Dr. L. Cohen, LC-NIAMD (Serial No. 42)
Dr. H. Eny, LC-NIAMD (Serial No. 37)
Dr. D. C. Gajdusek, NINDB (Serial No.)
Dr. E. Weinbach, LTD-NIAID (Serial No. 41)
Dr. Karl A. Schellenberg, LTD-NIAID (Serial No. 34)
Dr. J. E. Folk, ORC-NIDR (Serial No. 39)
Dr. E. Mihalyi, LCPM-NHI (Serial No. 127)
Dr. B. Horvath, MM-NINDB (Serial No. 6 [illegible])
Dr. Leo Mandelkern, Natl. Bur. of Standards
Dr. F. C. Baxter, GMET-NHI (Serial No.)
Dr. E. Eumart, LPT-NIAMD (Serial No. 102)

Man Years (calendar year 1959): Total: 20-1/3
Professional: 13-1/3
Other: 7

Project Description:

Objectives:

The chief interest of this section remained the study of the correlation between structure and function.

The problem of structure in its relation to function is very explicit in processes where one form of energy is converted to another form. In a different aspect, structure and function are the characteristic features of the mode of action of enzymes. Both of these aspects of structure and function have been under continued study in this section.

Muscular contraction is an example where chemical energy is converted into mechanical work. A great deal of study was devoted toward the understanding of the contractile muscle proteins (myosin, actin, tropomyosin) and their interaction with ATP. During this year studies have been made on the enzymes--thrombin, carboxypeptidase A, B, myosin ATPase.

The polymerization of proteins remained under study in order to gain insight as to how cells build up network structure such as the structures involved in muscular contraction and blood coagulation.

Methods:

The method of attack is both direct and indirect. In the direct attack, for example, the proteins of muscular contraction and blood coagulation are separated from their native milieu and are studied under arbitrary conditions which are selected to reveal properties of interest. In the indirect attack studies are made on some other (already better) known proteins to gain information before the direct attack is made.

In these studies the procedures of biochemistry and physical chemistry are employed. For example: paper and ion-exchange chromatography, enzymology, ultracentrifugal analysis, osmometry, light scattering measurements, electrophoresis, diffusion measurements, X-ray diffraction, etc.

Major Findings:

Muscular contraction - X-ray diffraction studies revealed that during the ATP induced contraction of glycerol-treated muscle fibers the α-keratin pattern disappeared, indicating that the basic process in contraction is the "melting" of the ordered filaments of the contractile proteins.

Major Findings: (Cont'd.)

These findings assign two roles to ATP. The contraction is initiated by ATP. In this process the energy of ATP is not used up. In a sense it acts as a signal and the contractile structure acts as an "amplifier." ATP also acts as the ultimate energy source by restoring the contracted structure to its original state. The immediate source of the contraction then is the "tendency" of the ordered regions of the contractile structure to go to the random form ("melting").

The method of analysis of magnesium in serum has been successfully extended to ascertaining the magnesium content of myosin B. (Mandelkern, Posner, Diorio, and Laki).

Polyphosphate $(Na_3PO_3)_{14-20}$ has been found capable of causing rapid relaxation of glycerol-treated muscle fibers which have been made tense by application of ATP. Several parameters of this phenomenon were studied. (Bowen, Martin).

Myosin -- It is now becoming increasingly apparent that without the understanding of the detailed composition of myosin, its role in muscular contraction cannot be evaluated.

Myosin has been isolated from earthworm and some of its properties studied. (Kominz, Maruyama).

Myosin preparations from normal and from failing hearts of dogs show the same sedimentation and diffusion constants and hence the same molecular weights. (Connell in cooperation with J. O. Davis).

Identification of myosin as a major constituent of the conduction bundle ("bundle of His") of beef heart was established by sedimentation and electrophoresis studies. The other protein constituents in an unfractionated extract are very similar to muscle, even though this tissue is thought to have a primarily nervous function. (Helander, Mitchell).

The strongly acidic (because of the presence of cysteic acid) phosphate-containing peptides obtained from myosin by partial acid hydrolysis were further characterized (Laki, Khalyl, Kwoller).

Actin -- The arginine-containing peptides obtained from actin by the action of trypsin are under study. The nature of these peptides [illegible] in determining the [illegible] of the [illegible] action from that actin. In one of the [illegible] (Laki)

Major Findings: (Cont'd.)

The C-terminal end group of rabbit actin was quantitatively determined by using carboxypeptidase A. (Laki, Standaert.)

Optical rotatory dispersion studies on actin gave strong indications that on G-F transformation the $(\alpha)_D = -57^\circ$ of G-actin changes to -31°. Since in 6 M urea both G- and F-actin give $(\alpha)_D = -100^\circ$, the change is interpreted to indicate a gain in order for F-actin. (Laki, Standaert).

Heat measurements made with the Benzinger micro colorimeter during G- and F-actin transformation indicate that ΔH for the change of the G-protein to F-protein is about + 2000 cal. This observation combined with the optical rotatory measurements indicate that the G-F transformation (at least in 0.1 M KCl) is driven by the energy liberated from ATP. (Laki, Kitzinger).

Structural studies on other proteins:

Salmine: The isolation of peptides formed during partial hydrolysis of salmine has been accomplished, and the composition of some determined in an attempt to learn the sequence of amino acids in the protein. (Knoller and Carroll.)

The structural details of the protein molecule that can be revealed by the study of the binding of cations and anions have been under continued investigation.

The binding of Ca^{++}, H^+, and Cl^- to serum albumin has been studied and the nature of the electrostatic effect determined. (Saroff, Lewis.)

A study comparing the binding of anions to the protonated nitrogen in model compounds and proteins is almost completed. The binding of chloride ions to detergents in the monomeric and micellar forms revealed a much lower electrostatic effect than that predicted indicating a high water content and consequent shielding of charges in the detergent micelle. (Saroff, Healy).

The study of the complexation of anions with salmine gave constants for binding which were unusually high (compared to detergent micelles) indicating a clustering of charges in a manner such that the water molecules are not shielding the charges as effectively as in the soap micelle. In addition, the quantitation of the binding of anions to salmine revealed a structure for salmine which groups the arginine residues into six clusters of three residues each.

Major Findings: (Cont'd.)

This structure is compatible with the aggregation of salmine with its associated nucleic acid. (Carroll, Saroff).

At present under study is the binding of anions (chloride and bromide) to lysine and polylysine (obtained from M. Sela, Weizmann Institute, Isreal). These results will be compared to those obtained from albumin, the detergents, and salmine. (Saroff).

Anion binding studies on salmine combined with the amino acid analyses of salmine and studies on its size and shape have prompted us to consider the implications of the observed heterogeneity of salmine. An analysis of this heterogeneity has been undertaken to show that there is a possibility that genetic information is transmitted by the salmine molecule. (Saroff, Carroll).

A continued study is being carried out on the relationship of the SH group to specific structures in the protein molecule. At present under study is the relationship of the SH group to the heme function in hemoglobin. The SH content of hemoglobin was found to vary with the pH. (This probably explains some of the disagreements in the literature on the SH values of hemoglobin.) Kinetic studies on the decrease of the SH titre of hemoglobin have been encouraging so far since the rate of decrease is about one order of magnitude faster than that previously found in serum albumin. Mercury in the bi- and monovalent form as well as silver are being used in our analyses to remove the ambiguity resulting from the use of bivalent cations alone. An attempt will be made to correlate the decrease in SH with pH with the Bohr effect (change in pH with O_2 binding) and with the reversible dissociation of hemoglobin. We are speculating on the role of the thiazoline ring in this reaction. (Saroff, Simpson).

Collagen -- In the course of studies on collagen metabolism in health and disease, a dipeptide containing an equal amount of proline and hydroxyproline has been isolated from human urine. This compound corresponds by chromatography and color reactions with a synthetic L-prolyl-L-hydroxyproline. This compound in human urine appears to be the major form of hydroxyproline excretion. (Irreverre).

From the hydrolysate of an antibiotic, telomycin, a new cyclic imino acid has been isolated which by chromatographic behavior may possibly be a 3-hydroxyproline. To characterize this compound 3-hydroxyproline is being synthesized. (Irreverre, Cohen).

Major Findings: (Cont'd.)

Studies on the changes in physical properties of serum albumin under various storage conditions for times up to 5 years have continued. There is good correlation among changes in ultracentrifuge pattern, viscosity, and reaction with trichloracetate with time and temperature of storage. The decreased solubility in dilute trichloracetate is a promising indicator of physical changes and has interesting aspects. (Tripp and Carroll).

Enzymes:

Thrombin -- Thrombin has been purified via cellulose exchangers. Preliminary studies show that working below pH 6.5 and above pH 7.2, the enzyme is soluble. This enables us to study the kinetic-molecular properties of thrombin. (Gladner, Folk, Laki).

Preliminary studies indicate that by the action of the Laki-Lorand factor only one peptide is liberated from fibrinogen by thrombin. (Gladner, Loewy, Laki).

A number of peptides of arginine were prepared for studying the specificity of thrombin.

Gly.Arg.Am. and Phe.Arg.Am. are split by thrombin very slowly. Elongation of the chain from the C-terminal does not seem to influence the rate of the reaction, as Gly.Arg.Gly. Et is split in the same magnitude of order of velocity as the amides. On the other hand, change in the N-terminal does influence the velocity of the peptide splitting by thrombin. By blocking the amino group, the peptide is split considerably faster, e.g., Bz.Gly.Arg.Am., and Bz.Gly.Arg.Gly.Et. are split much faster than the corresponding Gly.Arg.Gly.Et. and Gly.Arg.Am. Elongation of the peptide on the N-terminal has the same effect, e.g., Phe.Gly.Arg.Am. and Gly.Phe.Arg.Am. are split much quicker than the corresponding Gly.Arg.Am. and Phe.Arg.Am.

Thrombin was found to have wide esterase activity. Bz.Lys Me. is split quite quickly, about one-fifth the rate of splitting of Bz.Arg.Et. Addition of soybean inhibitor did not change the velocity of the reaction which shows that the splitting of Bz.Lys.Me. is not due to contamination of thrombin by plasmin. Benz.Gly.Lys.Me. is split even faster. Bz.Ornt.Me. and Cbz.Gly.His.Me. are also split by thrombin. It seems that the esterase activity of thrombin is toward all the basic amino acids. (Levin).

Major Findings: (Cont'd.)

Carboxypeptidase -- Preliminary studies on carboxypeptidase A have shown that the enzymatic activity of this metallo-protein (zinc) can be further enhanced by the addition of cobalt ions, increasing the activity as much as 100%. The reaction is pH, temperature and concentration dependent. Whether or not the effect is due to replacement of zinc by cobalt or cobalt entering a second "active site" is under investigation. (Gladner, Folk, Smith).

Carboxypeptidase B has now been isolated in highly purified form from pig pancreas. It appears from hydrodynamic measurements to have a molecular weight of 34,000. It appears to be a metallo-protein containing zinc. Although its specificity differs markedly from the well known carboxypeptidase A (above), its similar molecular properties to this enzyme is remarkable (Gladner, Folk, Carroll).

Trypsin -- Under proper conditions, trypsin can bind a second molecule of DFP. Using DFP^{32} to bind to this second site, we have been able to isolate a peptide (19 amino acids) whose amino acid analysis shows it to differ from the site of the first DFP-binding. Since binding the first site with DFP inhibits the enzyme, it is of great interest to elucidate the complete structure of the second site. (Gladner, Viswanatha).

The entire sequence of peptide A liberated from fibrinogen during clotting has been elucidated. (Gladner, Folk, Levin).

Enzyme complexes -- In collaboration with Professor Lester J. Reed of the University of Texas, studies have been carried out on the hydrodynamic properties and size of two large enzyme complexes isolated from bacteria: α-ketoglutarate dehydrogenase complex and pyruvate dehydrogenase complex. These have molecular weights of 2.4 and 4.4 million respectively, and behave as fairly compact spheres as judged by sedimentation, diffusion, and viscosity measurements. Each complex is capable of carrying out four to six enzymatic steps in the oxidation of substrate, and contains the appropriate co-enzymes in fixed amount. Studies of the fragmentation of the complexes in ways to maintain separate activities has been started. (Carroll).

New amino acid -- Studies on the detection, isolation, and characterization of nitrogen compounds (related to amino acids) in living systems.

A new acidic aromatic amino acid has been isolated α(m-carboxyphenyl) glycine from iris bulbs (Iris tingitana var. Wedgewood). (Irreverre, Thompson, Asen).

Major Findings: (Cont'd.)

This amino acid has also been synthesized and the N-acetyl and N-chloroacetyl derivatives prepared. Enzymatic studies were made on these compounds with the view of separating the stereo-isomers.

It was found that the urines of normal infants contain an α-amino acid which does not correspond to any α-amino acid known both naturally occurring and synthetic so far studied. It was established that this amino acid did not come from the food ingested. And it does not occur in the urines of older children and adults (over 100 examined).

The examination of the urines of EURU cases from the wilds of New Guinea for amino acids and total nitrogen showed very interesting and unusual patterns. This work is still in progress (Irreverre, Gajdusek).

Significance:

When part of the protoplasm or the whole cell (as in, e.g., cell division or muscular contraction) performs mechanical work, a network structure is built up at least temporarily, mainly through an orderly polymerization of globular proteins. This structure then reacts with the surrounding medium and by utilizing metabolic energy (stored in ATP e.g.) performs work (muscular contraction, amoeboid movement). In order to understand this "mechano-chemical coupling" (the interaction of structure with the surrounding and its disorders) we must know how such structures are built up. In addition we also have to know the detailed structure of the "building stones," the structure of the polymerizing proteins. Muscular contraction and blood coagulation are examples of processes where structures are built up through protein polymerization. Such knowledge eventually will lead us to the understanding of certain diseases of muscle. Study of blood clotting, in addition to supplying clues for protein polymerization, gives us better understanding of the disorders of blood clotting.

When both direct and indirect approach leads to some specific disease (e.g. hemophilia, rheumatoid arthritis) the advantage offered by studying the disease is utilized to the extent profitable.

Proposed Course of Project:

In the next calendar year we will follow in logical sequence the topics outlined.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Bowen, W. J., and Martin, H. L.: Analysis of myosin B for magnesium. Arch. Biochem. and Biophys., in press.

2. Bowen, W. J., and Martin, H. L.: Analysis of serum magnesium in presence of calcium with Chrome Fast Blue BG. Soc. Exptl. Biol. and Med. 101: 734-736, 1959.

3. Carroll, W. R., Callanan, M. J., and Saroff, H.A.: Physical and chemical properties of protamine from the sperm of salmon (Oncorhynchus Tschawytscha). II. Anion binding characteristics. J. Biol. Chem. 234: 2314-2316, Sept. 1959.

4. Folk, J. E., and Gladner, Jules A.: Carboxypeptidase B. III. Specific esterase activity. Biochim. Biophys. Acta 33, 570-572, 1959.

5. Gladner, Jules A., Folk, J. E., Laki, K., and Carroll, W. R.: Thrombin-induced formation of co-fibrin. I. Isolation, purification and characterization of co-fibrin. J. Biol. Chem. 234: 62-66, Jan. 1959.

6. Folk, J. E., Gladner, Jules A., and Laki, K.: The thrombin-induced formation of co-fibrin. II. Preliminary amino acid sequence studies on peptides A and B. J. Biol. Chem. 234: 67-70, Jan. 1959.

7. Folk, J. E., Gladner, Jules A., Viswanatha, T.: A simplified chromatographic purification of leucine aminopeptidase. Biochim. Biophys. Acta 36: 256-257, Nov. 1959.

8. Folk, J. E., Gladner, Jules A., and Levin, Y.: Thrombin-induced formation of co-fibrin. III. Acid degradation studies and summary of sequential evidence on peptide A. J. Biol. Chem. 234: 2317-2320, Sept. 1959.

9. Evans, R. L., and Irreverre, F.: Synthesis of γ-amino-butyryl-γ-amino-butyric acid. J. Organic Chem. 24: 863, 1959.

10. Irreverre, F., and Terzian, Levon, A.: Nitrogen partition in the excreta of three species of adult mosquitoes. Science 129: 1358-1359, May 15, 1959.

Publications: (Cont'd.)

11. Irreverre, F., and Evans, R. L.: Isolation of γ-guanidinobutyric acid from calf brain. J. Biol. Chem. 234: 1438-1440, June 1959.

12. Kominz, D. R., Carroll, W. R., Smith, E. N., and Mitchell, E. R.: A subunit of myosin. Arch. Biochem. and Biophys. 79: 191-199, 1959.

13. Maruyama, K., and Kominz, D. R.: Earthworm myosin. Zeitschrift fur vergleichende Physiologie 42: 17-19, 1959.

14. Saad, F., Kominz, D. R., and Laki, K.: A study of the tropomyosins of three cold-blooded vertebrates of different classes. J. Biol. Chem. 234: 551-555, Mar. 1959.

15. Laki, K., and Standaert, J.: The minimal molecular weight of actin estimated with the use of carboxypeptidase A. Arch. Biochem. and Biophys., in press.

16. Mandelkern, L., Posner, A. S., Diorio, A. F., and Laki, K.: Mechanism of contraction in the muscle fiber ATP system. Proc. Natl. Acad. of Sciences 45: 814-819, June 1959.

17. Saroff, H. A., and Healy, J. W.: The binding of chloride ions to alkyl amines. J. Phys. Chem. 63: 1178-1181, 1959.

18. Saroff, H. A.: On the acyl shift in protein reactions. Enzymologia 21: 101, 1959.

19. Saroff, H. A., and Evans, R.L. Chem. Biophys. Acta 36: 511-518, Dec. 1959. The conversion of the amino group of amino acids and proteins to the non-basic nitroguanidino group. Biochim.

20. Helander, E., and Emmart, E. W.: The localization of myosin in the conduction bundle of the beef heart. Proc. Soc. Exptl. Biol. and Med. 101: 838-842, 1959.

21. Folk, J. E., and Gladner, Jules A.: Cobalt activation of carboxypeptidase A. J. Biol. Chem., in press.

22. Asen, S., Thompson, J. F., Morris, C. J., and Irreverre, F.: Isolation of β-aminoisobutyric acid from bulbs of Iris Tingitana var. Wedgewood. J. Biol. Chem. 234: 343-346, Feb. 1959.

23. Morris, C. J., Thompson, J. F., Asen, S., and Irreverre, F.: Isolation of a new acidic aromatic amino acid, α(m-carboxyphenyl) glycine from Iris bulbs. J. Am. Chem. Soc. 81: 60-69, Nov. 1959.

24. Irreverre, F., and Levenbook, L.: The effect of diet on the free amino acid patterns in the blood of southern army worm (Prodenia Eridania). Biochim. Biophys. Acta, in press.

Serial No. NIAMD -109
1. Physical Biology
2. Physical Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Immunochemical approaches to the isolation and characterization of proteins

Principal Investigator: Dr. R. E. Williams

Other Investigators: None

Cooperating Units:

Dr. E. M. Lerner, LPH-NIAMD (Serial No. 82)
Dr. K. J. Bloch, A&R-NIAMD (Serial No. 1220)

Man Years (Calendar Year 1959): Total: 1

Project Description:

Objectives:

Production of experimental arthritis and abnormal serological reactions in animals.

Methods:

Sensitized sheep cell agglutination, ultracentrifugal analysis, and chemical analysis of proteins.

Major Findings:

The observation that rats injected with Streptobacillus Moniliformis develop joint lesions and positive sensitized sheep cell agglutinations has been extended. The serological reactions of rats and rabbits to killed organisms have been studied and found (positive B.F.T. reaction) to be largely due to immunization with gamma globulin in human ascitic fluid. The immunization of rabbits with killed organisms grown in media containing human ascitic fluid produced high BFT titres, but no elevation in SSCA titre.

Significance:

Positive flocculation tests in experimental animals must be carefully evaluated.

Proposed Course of Project:

Extension and confirmation of above.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Lerner, E. M. II, Bloch, K. J., and Williams, R. R., Jr.: Rheumatoid serological reactions in experimentals. II. Bentonite flocculation tests in rats with experimental arthritis. Arthritis and Rheumatism, in press. (With technical assistance of Marion Robertson, Ralph T. Groomes, and Clarence C. Israel.)

Serial No. BIAGB-110
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Investigation of the macromolecular organization of living matter.

Principle Investigators: R. W. G. Wyckoff (retired August 1959) and L. W. Labaw

Other Investigators: V. M. Mosley, G. Cerlin (Visiting Scientist from January through May 1959)

Cooperating Units: Pierre Lépine, Institute Pasteur, Paris, France (through May); D. B. Scott, National Institute of Dental Research

Man Years:
Total: 4 1/2
Professional: 3
Other. 1 1/2

Project Description:

Objectives: To gain information about the macromolecules that are essential constituents of living matter, to see how they are arranged in the structures they form and to see how this arrangement is altered by infections and degenerative disease. To study certain of these macromolecules, such as viruses and other proteins, in purified form after isolation from the living material. To improve the resolution of the electron microscope and to interpret the way in which images are formed near the limit of resolution.

Methods Employed: The electron microscopy of microorganisms, cells and tissues in suspension or thinly sectioned. The physicochemical characterization of macromolecular components isolated from such material using electron microscopy, X-ray diffraction, and similar established techniques; the development of new physical procedures, including X-ray microscopy and long wavelength X-ray diffraction, to further such characterization.

Major Findings: (1) The analysis of the crystal structure of protein crystals by means of electron micrographs and the use of models has been continued. The crystal structure of the Rothemsted tobacco necrosis protein has been determined in this way and is in close agreement with the crystal structure found using X-rays.

(2) The photography of the molecular separations in crystals of organic compounds of molecular weights 500 to 700 has been continued. It has been determined that this is not a direct imaging of the crystal planes but is rather an interference pattern produced by phase changes in the electron waves between those passing in between the properly oriented crystal planes and those passing thru these planes. This interference pattern can have the same spacing as the molecular plane spacing determined by X-ray diffraction, but appears above and below focus rather than in focus. The fine structure of the interference pattern, including halving of the spacing for some positions of focus, can be predicted and checks with the experimental data.

(3) The micro-spot X-ray microscope has been adapted to photograph diffraction patterns using long wave-length X-rays up to 10 Å. The resulting dispersion on the recording plate has been increased, for short plate distances, beyond that possible with commercially available X-ray diffraction apparatus. This permits the easy determination of large molecular plane spacings in crystals.

Significance to the program of the Institute: There is increasing interest in imaging and interpreting fine structure using electron microscopy. To interpret electron micrographs of some of these fine structures accurately it is necessary to go back to considerations of basic image formation, particularly for structures less than 50 Angstrom units in size. It can be shown, for instance, that periodic structures in this range can produce interference images which have periodicities other than those in the object, depending on the condition of focus. Periodicities other than those of the object can also be present if Bragg reflections from the object contribute to the image. It is believed important to try to define the conditions under which these artifacts take place as an aid to the interpretation of the fine structure appearing in electron micrographs.

Proposed course of the Project: The investigation into the use of characteristic X-ray absorption and fluorescence to

localize elements in sections photographed with the X-ray microscope will be continued. The use of this instrument adapted for diffraction together with standard X-ray diffraction apparatus will be used to further the work outlined in the previous paragraph.

Part B. included, Yes

PHS-NIH
Individual Project Report
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Part B.

Publications other than abstracts from this project

Labaw, L. W. and Wyckoff, R. W. G.: The electron microscopy of tobacco necrosis virus crystals. J. Ultrastructure Research 2: 8-15, 1958

Labaw, L. W.: An electron microscope investigation of tobacco necrosis virus crystal structure. J. Ultrastructure Research 2: 177-184, 1958

Wyckoff, R. W. G. and Labaw, L. W.: Infectivity studies of bacteriophage on several induced mutant strains. Proceedings of the 4th International Congress on Electron Microscopy, West Berlin, Germany, September 1958

Labaw, L. W.: An electron microscope investigation of influenza A virus crystals grown in the chick allantoic cavity. J. Ultrastructure Research [illegible]

Hyman, H., Beard, D. B., Labaw, L. W.: [illegible] Journal of Hygiene [illegible] press

1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The physical chemistry of membranes and complex membrane systems of biological interest.

Principal Investigator: Karl Sollner.

Other Investigators: Ruth McClintock (left August 31, 1959); Gerald H. Shean; and Stanley D. James (since April 14, 1959).

Cooperating Units: Loose cooperation is maintained with Dr. Charles W. Carr, Associate Professor, Dept. of Physiological Chemistry, Medical School, Univ. of Minnesota, and with Dr. Eugene Grim, Associate Professor, Dept. of Physiology, Univ. of Minnesota.

Man Years (calendar year 1959):

Total:	3-1/3
Professional:	3-1/3
Other:	0

Project Description:

Objectives: A physicochemical study of membranes and membrane model systems with the purpose of providing a rational physicochemical basis for the elucidation of numerous phenomena in living organisms, for instance, electrolyte balance and electrolyte distribution, the accumulation of electrolytes in living cells, cell and nerve potentials, and electrophysiology in general.

Methods employed: The preparation of porous membranes of highly characteristic and specific electrochemical properties (the methods having been worked out by the principal investigator and his collaborators), and recently also of oil membranes of somewhat similar characteristics and the investigation of these membranes, and of membrane systems in which such membranes are functional parts, by physicochemical, especially electrochemical, methods, such as potential and resistance measurements, also by chemical analytical procedures, including radioactive tracer methods.

Major findings: Theoretical considerations had led to the prediction that the ratios of the rates of the electrical transportation across permselective membranes of any two species of ions of the same charge coexisting in solution, should be predictable quantitatively, a) from the bi-ionic potentials arising with the same ions across the same membrane, and b) from the ratio of the rates of the exchange of the same two ions across the same membrane against a third ion. The experimental results were in fair agreement with the predictions. However, significant deviations, outside of the range of the experimental

Major findings (cont'd.):

errors occur regularly which must be assumed to be due to electrophoretic interaction between ions of the same charge, the solvent, and the pores of the membrane when an electric current is sent through the system. At higher current densities the situation is still more complicated by polarization. For the time being it seems that no reasonable amount of experimental work could clarify in detail these highly involved problems, the further investigation of which has, therefore, been discontinued.

Significant progress has been made in the study of "oil" membranes, particularly by the use of porous "Teflon" discs which are filled with the oil. This arrangement supplants the traditional U-tube in which the thickness of the "membrane" is of the order of 10 cm. The new technique reduces the resistance of the experimental cells by two orders of magnitude and correspondingly accelerates the rates of ionic exchange across them by the same factor. Some newly developed commercial preparations, "liquid ion exchangers", were found to be promising active molecular species to be incorporated in the membranes. Their properties seem to simplify the system significantly compared with those of prior authors. On the basis of much tedious and time-consuming preliminary work it seems now justified to express the hope that we will be able to make, over a period of years, substantial progress in a field which mainly for the experimental difficulties involved has been more or less dormant for several decades.

The concentration potentials which arise in cells with permselective membranes and electrolytic solutions agree closely in a medium range of concentrations with those calculated from known data on the basis of conventional assumptions for cells with membranes of ideal ionic selectivity. The experimentally determined potentials at low concentrations (smaller than about 0.04N) are consistently below the theoretical values, the discrepancies being larger the lower the concentrations. It was demonstrated that this unexpected effect is not due to imperfections of the membranes. Theoretical considerations ruled out the possibility of a major importance of osmotic water movement but have led to the hypothesis that membrane hydrolysis might conceivably account for the observed effect.

The fact that water is an ionizing liquid and that the distribution of the H^+ and OH^- ions across the membrane must under equilibrium conditions (under which alone the conventional theory applies) conform to the postulate of the theory of the Donnan membrane equilibrium

$$\frac{a_{H^+}^{(1)}}{a_{A^+}^{(1)}} = \frac{a_{H^+}^{(2)}}{a_{A^+}^{(2)}} \quad \text{or} \quad \frac{a_{OH^-}^{(1)}}{a_{B^-}^{(1)}} = \frac{a_{OH^-}^{(2)}}{a_{B^-}^{(2)}},$$

has been hardly regarded in the recent literature on membrane potentials. Cells in which the Donnan condition is not fulfilled are not equilibrium (or quasi-equilibrium) systems in which alone the conventional way of calculating theoretical values of these potentials could be strictly valid. From the theoretical point of view conventional aqueous concentration cells with permselective membranes have to be considered as dynamic "two ionic" cells to which the dynamic theory of polyionic potentials, as developed recently in this laboratory, must be applied. It was shown semi-quantitatively that experimental cells set up originally with two neutral solutions drift slowly, by the exchange of ions between the two solutions, towards a distribution of the ions of the water which corresponds to the Donnan equilibrium, the deficiency in potential in such cells being due to a kind of internal short-circuiting. It also could be shown semi-quantitatively that pairs of solutions adjusted beforehand to the proper ratios of hydrogen ion concentrations yield potentials much closer to the theoretical (reversible) values than those of cells with solutions of equal hydrogen ion concentration. The experimental difficulties in obtaining quantitatively satisfactory data for publication are considerable due to the extreme pH sensitivity of the unbuffered experimental solutions near the neutral point; work along these lines is in progress. In principle, however, the before-mentioned discrepancies between calculated and theoretical potential values in membrane concentration cells seem to be resolved. These results have obvious bearings on the evaluation of experimental potentials in many systems involving membranes, including cells in which permselective membranes are used as membrane electrodes.

Experimental work on an improved model for the accumulation of electrolytes, of anions and of cations simultaneously (Science, [illegible] 939 (1956), once more has confirmed the correctness of the previously developed theory. The rather involved experiments necessary in these tests will require a great deal of additional work before the manuscript will be ready for publication.

Significance to Research of the Institute: In order to understand electrolyte relationships in living cells and tissues, it is necessary to have accurate information on membrane model systems which under carefully controlled known conditions, reproduce at least some of the major *in vivo* phenomena. The work of recent years, particularly the study of polyionic potentials, of absolute and relative rates of ionic fluxes under various conditions, and the construction of an *in vitro* model of electrolyte accumulation have brought us significantly nearer to an understanding and an *in vitro* reproduction of the type of effects which ultimately must govern the *in vivo* osmotic behavior of cells and tissues. The work already carried out indicates that even fairly complex membrane systems, similar to those found in living nature, may prove in the foreseeable future amenable to a complete and quantitative physicochemical analysis.

Proposed Course of Project: Further experimental work on electrolytic accumulation against concentration gradients. Further work on membrane hydrolysis and its influence on membrane potentials. Resumption of the work on the absolute rate of exchange of ions across permselective membranes, from the experimental and theoretical point of view. Accelerated continuation of the studies on oil membranes on the basis of the above described results. Over the long range many of the effects studied with permselective membranes over the last 20 years, such as their use as membrane electrodes, or in the study of membrane equilibria, or in the investigation of ionic specificities, etc., should also be investigated with oil membranes, as far as they lend themselves satisfactorily to these purposes.

In addition, largely depending on the availability of a suitable collaborator a study is planned of the forces which operate in the spontaneous formation of regular structures of microscopic and sub-microscopic (but greater than molecular) dimensions. Long range forces of attraction and repulsion between microscopic and sub-microscopic particles are known to exist. These forces are accessible to quantitative measurements by methods developed by the senior investigator before coming to NIH. These studies are designed to furnish an insight into the physical forces which create organized structures of various levels of complexities as those existing in living systems.

Is B included? Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Lewis, Marc and Sollner, Karl: Preparation and Properties of Improved Protamine Collodion Matrix Membranes of Extreme Ionic Selectivity. J. Electrochem. Soc. 106, 347-354 (1959)

McClintock, Ruth, Neihof, Rex, and Sollner, Karl: The Relative Rates of Electromigration of Different Ions of the Same Charge Across Permselective Membranes. J. Electrochem. Soc. (in press).

Serial No. NIAMD - 112
1. Physical Biology
2. Photobiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar year 1959

Part A.

Project Title: General Project Domain of Section - Molecular Mechanisms of Radiant Energy Transformation in Biological Structure.

Principal Investigator: F. S. Brackett

Other Investigators: (by projects - a to g).

(a) R. A. Olson, C. L. Greenblatt and E. Engel
(b) N. E. Sharpless, O. S. Temmer, and J. R. Hilla
(c) E. D. Becker and R. B. Bradley
(d) U. Weiss and H. Ziffer
(e) H. Charney and G. R. Gauvin (A. Shannon-summer emp
(f) W. A. Hagins
(g) E. G. Adams

Supporting Activities:

Electronic Development - Mr. Lawrence Shonkein
Instrument Development - Mr. Charles E. Lohr, Jr.
Data Processing - Mr. Wm. E. Hahn, Jr.
Secretary - Carmelia M. Joy

Co-operating Units:

Department of Terrestrial Magnetism of the Carnegie Institution,
Naval Medical Research Institute,
Dr. C. S. Watson, University of Minnesota
NCI Radiation Branch,
Dr. Lewis J. Sargent (LC-NIAMD), NIAMD-57
Dr. J. M. Bobbit & Miss D. Hamessian, Connecticut Staff and Ohio State Universities,
Urner Liddel, Biomucleonics, NIAID, -130
Laboratory of Technical Development, Heart Institute (See Serial No.),
Dr. William Carroll, Physical Biochemistry, NIAMD -102

Man Years (calendar year 1959)

Total:	19
Professional:	13
Other:	6

Brief Report of Research in Photobiology (Cont'd)

Progress is for the most part to be found in the piece by piece assembly of pertiment findings, thus:

In Photosynthesis: The organization of pigment-protein molecules into a functional network may explain the extraordinary effectiveness of this "machine". (a) Partially reversible changes in bleaching are found and a dependence on oxygen demonstrated. The site of this action is related to the lamellar chloroplast structure. (b) Digestion of lipoid from the chloroplast allows the still intact layers to separate in fanlike fashion. Protein digestion, on the other hand, causes the layers themselves to collapse.

Bearing on the mechanism of photosynthesis, it is shown that anomalies in scattering by the pigment account for only a small fraction of the wavelength change from free pigment to in vivo condition and so may be indicative of its relationship to the organized structure.

Also, related to the role of chlorophyll in photosynthesis are our findings concerning porphyrin structures:

(a) The specific stoichiometric nature of the binding of copper-porphyrin to bovine serum albumin is to be contrasted with the lack of such binding to β-lactoglobulin.

(b) Still more physical evidence is found from Nuclear Magnetic Resonance concerning the "ring current" producing local magnetic fields within the molecule due to the induced circulation of π electrons about the conjugated porphyrin ring.

Many of the facts, ideas, interests, and inquiries concerning one photomechanism apply to others, though different, photosystems for energy absorption and transfer. Thus, the study of the basic mechanism of vision proves closely allied to our interest in the molecular mechanism of photosynthesis. Here again the primary photo mechanism is least well understood. Here again the efficiency of the system transcends anything in our experience. Physical theory is shown to predict the minimum electrical current (about 1000 charges/photon) that a photo receptor must produce in order to convey information to the brain. Experiments carried out in collaboration with the Naval Medical Research Institute on the photo receptors of the squid, yield values 750 electrical charges per incident photon in good agreement with the prediction.

Brief Report of Research in Photobiology (Cont'd)

The genetic changes produced by ultraviolet are especially interesting as this is probably the region of energy threshold and may show specific selectivity in mechanism as contrasted with the random effects of high energy radiation. Looking toward such investigation, a co-operative study (with Dr. Elkind, NCI, Radiation Branch) of the chromosomal alteration, as related to the levels of lethality in dose of ionizing radiation is being carried out along lines closely related to those developed by Puck.

Instrumental Projects:

1. DATA PROCESSING - Mr. Wm. E. Hahn, Jr.

Contract let to Airborne Instruments Laboratory for the construction of a logging system which will take data from laboratory recording systems (analogue) and convert the information to digital form recorded on magnetic tape in a form suitable for direct processing on our central IBM 650 Computer.

A machine for plotting data from paper tape on a 11" x 17" graph paper, has been assembled from purchased components.

An Add Punch has been ordered for producing paper tape, either by manual transcription or by automatic punching through a solenoid deck.

2. The double monochrometer previously reported has been completed to the point of preliminary runs for the purpose of cutting the linearizing cams which are now ready for refinement. Accessory equipment is still under construction.

3. The grating system for quantum determination at two or more wavelengths has been completed and the thermal control system is being constructed. (This project was delayed by the long period of building construction in the areaway.

4. Electrode technique for O_2 and CO_2 determination was again the subject of study. A new electronic approach was suggested by the study of Lessajous figures. A dual purpose membrane-protected flow electrode has reached a later stage of development (see Olson).

Essential to an understanding of these mechanisms is the role of the pigment which absorbs the energy and initiates the train of events which result in the conversion of chemical storage of energy on the one hand on the one stimulus on the other. In a widely diversified group as this, the behavior in these pigments, ranges from that of the structural organic chemist to physicists interested in triplet and metastable states which may be involved in the mechanism of energy transfer.

Thus, a vast pigment naturally synthesized by a wide variety of the genus Rhodospirillum has been isolated and studied chemically and physically intensively both because of its unusual spectrum of conjugation and also because of its photodynamic action. Pigments which produce such concentrations photo oxidation and sensitize to supply energy to valuable endothermic reactions when placed in a suitable setting as structural organisation in photochemical studies. The behavior of the light in important both because it governs the energy flow in the material system and also the phenomena of resonance transfer, the ultraviolet or high energy end of the spectrum predicates important chemical and biologic [illegible] not certain in this visible [illegible] comparison of 7-dehydro cholesterol [illegible] (Vitamin D_3), ergosterol, beta-sitosterol and cholesterol. [illegible] two have been studied in this group during the past [illegible]

Work on steroid photochemistry has been resumed in a threefold attack:

(a) Quantum requirements for transformation of ergosterol whose photochemistry is dominated by digitonin precipitable [illegible] being purified by a variety of [illegible] wavelengths in the ultraviolet immediately. This determination should mesh with data obtained by ultraviolet spectroscopy.

(b) Flash photolysis studies on mixtures of ergosterol and its isomers as well as pure compounds providing information on the nature of chemical steps of short duration.

(c) A re-examination of the great amount of data from an extensive study in monochromatic ultraviolet irradiation. It is shown that Velluz's concept of a photo steady state involving precalciferol can be extended to explain the remarkable wave length dependence of yield of tachysterol and other isomers. A dark reaction of precalciferol to tachysterol is suggested by our findings. This appears to be confirmed by the rate [illegible] found to follow flash photolysis.

Instrumental Projects: (Cont'd)

5. A spectral scanning device has been developed for microscopic study - yielding absorption, emission or fluorescent spectra or a time sequence at several wavelengths simultaneously. (see Olson).

6. An instrumental development has been completed for the study of chemical changes in Flash Photolysis. This has proved very fruitful in steroid photochemistry. (see Adams).

Improved instrumentation for Flash Photolysis is under construction, both for studies of vision (Adams and Hagins), and for steroid photochemistry (Adams with Sharpless and Brackett).

7. Other instrumentation for research on photoreceptor mechanism has been evolved. (see Hagins).

8. As a result of the co-operation with the Laboratory of Technical Development, Heart Institute, a "function generator" designed by that Laboratory has been constructed by the electronic shop and put into operation (This co-operation has been valuable in initiating other studies). This type of analogue analysis of our spectroscopic data is proving most interesting.

The project reported last year as: "Effect of Nuclear Radiation on Biological Systems" has become a new Section in the Laboratory of Physical Biology under the direction of Dr. Urmen Liddel. Dr. Liddel continues to collaborate in our studies of molecular structure and infrared spectroscopy.

Significance of the Program to the Institute:

Exploitation of nuclear energy has faced society with a group of serious hazards which are commonly referred to as caused by 'radiation'. Actually there is included a variety of causes - not only electromagnetic radiation but bombardment by particles of differing speed, mass, and charge.

Empiricism has provided some knowledge of the limits of "safe exposure" so far as immediate acute effects are concerned. The longer range implications of radiation damage, however, require not only experiments of long duration, but more insight into the basic nature of the action of radiation on living things.

Significance of the Program to the Institute:

The purpose of this section is to gain an understanding of these basic mechanisms at the molecular and cellular level.

Our primary concern is with electromagnetic radiation as contrasted with particle bombardment.

Furthermore, the region of more moderate energy (near visible) holds greater interest for us because of the resonance or correspondence of these frequencies to the mechanisms of biological structure. An understanding of these unitary processes may ultimately be extended to the random effects of "high energy radiation".

Our researches were undertaken because of their fundamental importance before nuclear energy and space travel focused public interest on this area.

Further planning beyond the scope of our present enterprises is limited by the serious lack of laboratory space and uncertainty of relief.

Serial No. NI[illegible]-113
1. Physical Biology
2. Photobiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Flash photolysis of Vitamin D and its precursors.

Principal Investigator: Ralph G. Adams

Other Investigators:

Co-operating Units:

Man Years (Calendar year 1959):
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: To elucidate the molecular changes accompanying the conversion of ergosterol, or any of the so-called intermediate compounds, to Vitamin D by ultraviolet radiation.

Methods Employed: The use of a short intense flash of ultraviolet light to which the compound under investigation is exposed produces changes in that compound's molecular patterns. These changes can be observed spectroscopically and, provided the flash duration is short enough, the kinetics of the subsequent processes can be measured. For this purpose, the flash is synchronized with an oscilloscope and camera, so the time course of any process occurring subsequent to the flash may be recorded. The samples are all maintained in an oxygen free state.

Major Findings: Thus far it has been determined that changes taking place as a result of exposure to the flash are the same as, and equivalent to in wavelength dependence, those resulting from classical steady illumination. It is apparent that there are changes taking place in the dark subsequent to reaching an equilibrium state by means of a series of flashes. These changes have been previously observed by Dr. Brackett but are now confirmed. There is a strong wavelength dependence of the equilibrium mix[illegible] which needs further investigation.

Significance of Research to the Institute: It seems sufficient to state that no satisfactorily understanding exists for physical mechanisms by which ergosterol is converted to Vitamin D. The explanation may well be found in the relation of steady state to dark reaction and photochemical efficiency.

Proposed Course of Project: Thus far the time resolution of present equipment does not allow sufficient investigation of the probable excited state of ergosterol and/or other intermediates. This equipment will shortly be greatly improved.

The dark reaction is at present under investigation and results will shortly be submitted in the form of a publication.

Part B included NO

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Molecular structure determined by spectroscopic methods.

Principal Investigator: Edwin D. Becker

Other Investigators: Robert B. Bradley

Co-operating Units: Dr. C. J. Watson, University of Minnesota (NMR Studies of Porphyrins)

Man Years (calendar year 1959):
Total: 2-1/3
Professional: 2
Other: 1/3

Project Description:

Objectives: An understanding of the forces within and between molecules, especially those of potential biological importance. (2) Development of spectroscopic methods for studying molecular structure and analyzing for materials of chemical and biological interest.

Methods Employed: The primary techniques used in this work are infrared spectroscopy (IR), nuclear magnetic resonance (NMR), and electron paramagnetic resonance (EPR). The IR and NMR methods supplement each other in providing detailed information about the structure of molecules. In addition, both types of spectra are highly sensitive to the effects of molecular interactions. EPR is used in studies of molecules having unpaired electrons, including free radicals and paramagnetic atoms and ions.

Major Findings: The project can be divided roughly into three parts: (a) studies of hydrogen bonding and other molecular interactions, utilizing both IR and NMR; (b) NMR studies of molecular structure in porphyrins; and (c) more general investigations of molecular structure by NMR and EPR:

Major Findings: (cont'd)

(a) We have made an IR investigation of hydrogen bonding between alcohols and various proton acceptors (e.g., acetone, pyridine, dioxane) in order to determine reliable values of equilibrium constants and energies of formation for such hydrogen bonds. These thermodynamic quantities are now being correlated with spectral properties, such as frequency shift and band width, in an attempt to provide more definitive criteria for the existence of hydrogen bonds and possibly improved methods whereby hydrogen bond energies can be estimated directly from spectral data.

(b) We have found that the NMR spectra of a series of metal-free porphyrin esters display unusual resonance frequencies for the methine and N-H protons. This behavior has been explained in terms of a "ring current" model, in which local magnetic fields are induced in the molecule by the circulation of π electrons about the conjugated porphyrin ring.

(c) A number of experiments have been carried out in collaboration with chemists in our laboratory and in other laboratories. NMR studies of several compounds (including derivatives of pyridine, codeine, dichlorobenzene and indole) have materially assisted in structural determinations. In an EPR study of the oxidation of chlorpromazine in vitro, we have demonstrated the presence of a free radical intermediate and have made some measurements on its rate of disappearance by further reaction.

Significance to NIAMD research: The further development of NMR and EPR is expected to be of considerable assistance to many NIAMD scientists, since these methods will add two more spectroscopic techniques that can be brought to bear on biological problems. For example, NMR spectra of such complex molecules as steroids and porphyrins are frequently helpful in unraveling their molecular structure. EPR studies may permit the detection of free radical or paramagnetic intermediates in reactions of biological significance.

A deeper understanding of the properties of hydrogen bonds is clearly desirable since such bonds are of prime importance in determining the structure and function of proteins and nucleic acids.

Proposed course of project: (a) Additional work on hydrogen bonding systems will be conducted along the lines already indicated. We are now planning NMR experiments to complement the IR results reported above, and are considering the extension of these studies to other spectral regions. (b) We are now interpreting

Proposed course of project: (cont'd)

the NMR spectra of porphyrins described above in order to obtain information on electronic structure and to provide a method of analysing for certain types of substituent groups on porphyrins. We plan studies with other metal-free and metal-substituted porphyrins. (c) We expect to continue our program of collaboration with other investigators in an effort to assist them in their analytical problem and to learn more about the molecular structure of interesting types of molecules. Specific problems already in progress (e.g., the free radical intermediate in chlorpromazine oxidation) will be continued with a view toward early termination.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Becker, E. D.: Infrared studies of hydrogen bonding in methanol, ethanol, and t-butanol. Symp. on hydrogen bonding, Ljubljana, Yugoslavia (July 29 to Aug. 3, 1957) pp. 155-162, 1959.

Becker, E. D.: NMR studies of hydrogen bonding in alcohols and phenol. J. Chem. Physics, 31: 269-270, 1959.

Becker, E. D. and Bradley, R. B.: Effects of "ring currents" on the NMR spectra of porphyrins. J. Chem. Physics, 31: 1413-1414, 1959.

Becker, E. D.: Infrared studies of the self-association of chloroform. Spectrochimica Acta, 9: 743-746, 1959.

Becker, E. D.: The effect of molecular interactions on NMR reference compounds. J. Phys. Chem., 63: 1379-1381, 1959.

Serial No. NIAMD - 115
1. Physical Biology
2. Photobiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Molecular Structure and Organization in Biologically Important Systems.

Principal Investigator: Elliot Charney

Other Investigators: Alice Shannon (summer employee)

Co-operating Units: None

Man Years (Calendar year 1959):
Total: 1-2/3
Professional: 1
Other: 2/3

Project Description:

Objectives: The general objectives of this research are to explore the nature of intermolecular interactions, the role they play in molecular organization and the relation of this organization to biologic activity.

Methods Employed: Two of the methods employed are somewhat unusual although related to techniques previously developed. In one of these the course of a protein-pigment complex during ultracentrifugation is followed by light absorption. This is done by photographing the precipitating protein-complex with the normal optics of the ultracentrifuge and with the normal filter replaced by a filter transmitting only in the spectral region absorbed by the pigment. The other method is concerned with testing theories of the apparent dipole moment of p-Quinone and involves examining the infrared spectrum of the gas of this compound under the influence of an electric field and the measurement of the electro-optic Kerr effect of a benzene solution of p-Quinone.

Major Findings: The strong stoichiometric complex which a copper-porphyrin forms with bovine serum albumin has been found to be stable over a pH range of 2-12 making it unlikely that this complex results only from the formation of salt bridges (the porphyrin in question has 3 carbonyl groups per molecule). The same porphyrin binds also to ovalbumin but does not form any complex at all with β-lactoglobulin. More recent results, not yet

Major Findings: (Cont'd)

completely analyzed indicate that, unlike the case of the copper porphyrin, the serum albumin complexes of heme and protoporphyrin are not stoichiometric in the same sense. A preliminary report of this work was given at the Biophysics Society Meeting, Pittsburgh, Pa., February, 1959, and a more complete report has been submitted for publication. In an effort to elucidate the role of copper in the complex studies are currently under way on the binding of two other porphyrins, chlorin e_4, chlorin e_6, and (hopefully) the copper derivatives of these compounds.

In collaboration with E. D. Becker, the infrared spectra of p-Quinone and completely deuterated p-Quinone in the gas phase and in solution have been taken at high dispersion and are being analyzed to assign all the spectral absorptions to the respective normal modes of these molecules. Other work on p-Quinone, which is the basis of the original interest in this molecule, has involved the test of theories of the origin of the dipole moment of this molecule by methods briefly described above. On the basis of the results thus far, it appears that, contrary to the literature, p-Quinone does not have a large permanent dipole moment, nor is it likely that such a moment is induced by the measuring field.

In collaboration with J. Weiss, the optical rotary dispersion of a number of compounds have been measured to wavelengths shorter than previously measured and anomalous behavior of the rotatary dispersion associated with chromophoric groups other than carbonyl has been observed. This work is more fully described in Dr. Weiss's report.

The data collected two years ago on the wavelength dependence of the scattering of light from a pelagical algae has been completely analyzed. The analysis shows that the long wavelength *in vitro* - *in vivo* shifts of the 680 mμ band of chlorophyll results only in very small part from light scattering and must, therefore, result primarily from the state of organization (crystallinity) or more likely from the *in vivo* complex of the chlorophyll to proteins. This work is complete and in manuscript.

Serial No. NBMB-116
1. Physical Biology
2. Photobiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: The physical and chemical basis of photoreception.

Principal Investigator: W. A. Hagins

Other Investigators: None

Co-operating Units: Naval Medical Research Institute, NNMC, Bethesda, Maryland

Man Years: (Calendar year 1959)
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: To outline the successive events by which light quanta absorbed in the receptors of animal eyes lead to the production of nerve impulse information to the brain.

Methods Employed: Two complementary methods have been used to explore the early stages in the response of retinal photoreceptors to light. In the first, the electric currents produced by receptors when stimulated by light have been measured with conventional electrophysiological techniques. In the second, absorption spectroscopy and flash photolysis have been used to study the photochemical reactions of the primary light-absorbing pigments in receptors.

Major Findings: A theoretical analysis has been made of the minimum electrical current that a photoreceptor must produce in order to convey information to the brain at the rate which has been observed experimentally by previous investigators. Using theorems from thermodynamic and information theory, it has been shown that photoreceptors of the usual range of sizes must produce electric currents of the order of at least a thousand electronic charges for each light quantum absorbed in the retinal photopigment.

Major Findings: (cont'd)

In collaboration with Dr. H. G. Wagner of the Naval Medical Research Institute, a direct experimental measurement of the current produced by photoreceptors of the squid in light of known absolute intensity has been made. It was found that the cells yielded at least 750 electronic charges for each incident photon. This result supports the conclusion of the theoretical analysis.

In order to extend these results and to investigate the process by which photoreceptors yield electric currents, apparatus has been built to measure currents, voltages, impedances, and radioactive tracer uptake by the retinas of octupus and squid and to study the photochemistry of their visual pigments in the living cells.

Significance of the program to the Institute:

A clear understanding of the physiological mechanism of light sensitivity in the retina may help to explain some aspects of the related problem of energy absorption and transfer in photosynthesis and cellular metabolism in general.

Proposed course of project:

The immediate objectives are to find answers to the following questions:

(1) Do the photochemical changes observed in visual pigments have anything to do with the physiological mechanism of light sensitivity? An attempt will be made to answer this by comparing the quantum efficiency of the photochemical reactions with that of physiological excitation.

(2) How do photoreceptors convert light into electric current? Using standard methods of electrochemistry and tracer technology, it is hoped that the ionic basis of receptor currents can be found.

Part II included Yes

Part E: Honors, Awards, and Publications

Publications other than abstracts from this project.

Hagins, W. A. and Jennings, W. H.: Radiationless [illegible] of electronic excitation in retinal rods. Faraday Discussions 27: 180-190, 1959.

NC1" 65/C

Methods Employed:

studies of the time course of changes occurring at relevant regions of the emission spectra, a disc bearing a series of interference filters was substituted for the rotating spiral slit. Each filter was provided with an accessory filter to exclude the exciting light. This type of scanning when displayed with a very slow (2 min.) horizontal sweep (time base) permitted as many as four simultaneous traces to be recorded from the oscilloscope, each showing the time course of changes in emission at wavelengths chosen. By substituting an appropriate filter, one of the four traces could be used to record changes in relative transmission. Hence, the time course of emission changes of cell structures at three spectral regions could be simultaneously recorded with the time course of bleaching.

The treatment of cell suspension, cell-free chloroplasts, etc., was as described in the previous report.

Major Findings: The performance of O_2- CO_2 hydrophobic membrane electrode developed in our laboratory appears to be adequate for application to a study of O_2 and CO_2 transients accompanying photochemical activity in cell suspensions. Its time response (less than one second) should permit a kinetic analysis of CO_2 and O_2 "bursts" etc., and "troughs". Furthermore, its immunity to "specific" enzyme inhibitors added to cell suspensions should permit identification of each successive transient with an appropriate metabolic step. In its present form after a few minor changes to enhance stability and response, it will be adopted in the near future to launch this heretofore impossible program.

Our development of the scanning spectrophotometric microscope has made it possible to follow, quantitatively, rapid changes in emission of fluorescent cellular structures and concomitant changes in transmission. At very high, but sublethal light intensities, chlorophyll fluorescence at 670 mμ at lamellar sites disappears and is replaced by yellow fluorescence peaking near 540 mμ. The effect is oxygen limited and is accompanied by a bleaching of the visible pigments. It can occur under ideal conditions at room temperature during one minute or less. Time scan studies of changes at various wavelengths of emission indicate the formation of a non-fluorescent intermediate. Attempts to identify the bright yellow fluorescent substance are based upon comparison with the emission spectra of known materials. At present photolytic oxidation products of chlorophyll are indicated and/or a flavin-like compound.

Major Findings:

Further studies with cell-free chloroplasts via absorption microscopy provide further interpretation of fine structure in vivo. Hypotonic swelling and the results of specific enzyme digestion produce characteristic alterations. Proteolytic enzymes disrupt the lamellar structure while lipolytic enzymes leave the organization intact. The pigment lamellae appear to depend on a protein substratum and are separated by an aqueous interphase.

Significance of Research to the Institute: The development and use of specialized optical and electrochemical instrumentation provides insight into processes of energy transfer which occur in the optimal biological material under study. Since the laminar fine structure appears to be common to other photoreceptor systems, the study of its role in photochemical metabolism leads to better interpretation of energy transfer in all living cells whether light or chemically activated.

Proposed Course of Project: Completion of the hydrophobic membrane electrode development opens the way to kinetic analysis of light induced O_2 and CO_2 transients. In addition, a systematic study of the effect of inhibitors on the numerous characteristic transients should aid in identifying their origin in the stepwise sequence of metabolic steps immediately following light or darkness.

Identification of the participating metabolites in the sequence of the fluorescence shift will be sought by comparison with fluorescence of pure compounds and by attempts to obtain large quantities of bleached cells for extraction analysis and chromatographic yield from extracts.

ADDENDUM:

Dr. C. L. Greenblatt has been collaborating with Dr. M. Elkind of the National Cancer Institute in a study of ionizing radiation in mammalian tissue culture cells for the latter half of the year. This work is an attempt to evaluate the role of chromosomal damage in cell lethality. The tissue being used is Chinese hamster, chosen for its low chromosomal complement (n = 11), and readily identifiable characters of the major chromosomes. These properties facilitate the determination of visible cytogenetic alterations.

Chromosome complement is being studied in cells just after irradiation as well as in cells which are long term survivors. The specific details of these observations as they relate to survival is referred to in Dr. Elkind's annual report (Cell Radiation Branch).

Page 7

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Olson, R. A. and Engel, E. K.: Visible absorption microscopy of pigment systems in living cells using interference filters: Chlorella Chloroplasts. Proc. Microscopy Symposium, Chicago, 1958, McCrone Assoc., 1959.

Olson, R. A. and Engel, E. K.: "Chlorophyll" absorption microscopy of in vivo, cell-free and fragmented Chlorella chloroplasts. Brookhaven Symp. on the Photochemical Apparatus, Its Structure and Function, Brookhaven Symposia in Biology, No. 11, 1958, 303, 1959.

Greenblatt, C. L. and Schiff, J. A.: A pheophytin-like pigment in dark-adapted Euglena gracilis. J. Protozool. 6: 25, 1959.

Greenblatt, C. L. and Sharpless, N. E.: Effects of some metabolic inhibitors on the pigments of Euglena gracilis in an acidic medium. J. Protozool. 6: 241, 1959.

Serial No. NIAMD-11B
1. Physiology
2. Photobiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Investigations of the Action of Radiant Energy on Biologically Important Compounds.

Principal Investigator: Norman E. Sharpless

Other Investigators: Odette S. Temmer

Co-operating Units: None

Man Years (Calendar year 1959)
Total: 2-1/4
Professional: 2
Other: 1/4

Project Description:

Objectives: The objectives of this project are the establishment of various biologically important intermediates in photobiology and the study of their kinetics and other physical and chemical properties.

Methods Employed: Ultraviolet or visible radiation is the means employed to effect any alterations in the material under investigation. Spectroscopy in the ultraviolet, visible and infrared regions, as well as chemical procedures, are the major methods used to evaluate any changes which occur.

Major Findings:

1. (With Mrs. Odette S. Temmer). The photochemistry of ergosterol and related steroids to form the various Vitamins D is one of the major photochemical reactions of biological importance.

Determination of the quantum requirements for the disappearance of ergosterol has been carried out as a function of both concentration and wavelength. Evaluation of ergosterol requirements in quanta per molecule has been carried out by determining residual ergosterol after irradiation by the digitonide procedure. Extrapolation to low doses of irradiation gives the quantum requirement

Major Findings: (cont'd)

corrected for the competition of the photoactive isomers formed. This quantum requirement ( ) has been further evaluated as a function of initial concentration of ergosterol. This value is in general a linear function of the initial ergosterol concentration. The final extrapolation to infinite dilutions gives the desired value, the quantum requirement of ergosterol corrected for extraneous light absorption and intermolecular factors ( ). A summary of the data so far obtained is:

Wavelength, A	
2650	1.6
2800	2.7
2967	3.1
3021	1.0
2537	2.9 @ 2.6 x 10^{-4} molar

Confirming values will be required for some of these figu[illegible]

2. (With Ulrich Weiss). The alkaloid thebaine has an absorption band at 285 mμ which has contributions from an aromatic ring and a conjugated diene system in the molecule. Irradiation of this compound in the ultraviolet region under anaerobic conditions causes this peak to drop to 50% of its value, presumably due to alterations in the diene system. Irradiation in the presence of air results in the same initial drop of intensity to the approximately 50% value, followed by a gradual disappearance of the band presumably caused by a photochemically induced oxidation of the aromatic system. The irradiated product is now under investigation to determine its structure.

Significance to research of the Institute: The proper understanding of the behavior of biologically important molecules on a molecular level is absolutely necessary to the extrapolation of their effects to a cellular level for the evaluation of their effects in health and disease.

Proposed course of project: Some more data is still required in the quantum yield of ergosterol disappearance as functions of wavelength and concentration. The reversibility of the ergosterol transformation will be investigated by irradiation of intermediates such as lumisterol and calciferol and analyzing for ergosterol.

The work on thebaine will continue, both to determine the nature of the product and the quantum efficiency of the process.

Part B included YES

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Part E. Honors, Awards, and Publications.

Publications other than abstracts from this project.

Greenblatt, C. L. and H. J. Sharpless: Effects of [illegible] Metabolic Inhibitors of Euglena gracilis in an Aquatic Medium. J. Protozool. 6: 241, 1959.

Major Findings:

that of 3, 10-dihydroxyperylene-4, 9-quinone, showing that the chromophoric part of the pigment molecule must be closely related to this quinone. The IR spectrum is in agreement with this conclusion. The pigments from Elsinoe are the first derivatives of perylene which have been found to occur in nature; the only derivatives of this ring system isolated previously from a natural source, the two erythrophins from aphids, do not occur as such in the insect, but are formed by enzyme action during isolation. Other representatives of this group have been found quite recently.

Work at WIM was first directed towards improved culture methods for Elsinoe, since growth and pigment production in the 2% malt extract solution previously used is slow and aeration by mechanical shaking is required; hardly any pigment forms in still cultures. However, all attempts to modify the medium gave results inferior to those obtained with 2% malt extract.

A satisfactory method for paper chromatography of the pigment complex was worked out and proved valuable in the subsequent investigations. Through its use it was found that the crystals obtained earlier by warming with [illegible] are chemically different from the native pigments; however the visible spectra of both types of compound are almost identical.

For separation of the complex into chemical individuals column chromatography using a variety of absorbents was not satisfactory. Countercurrent distribution gave more promising results, fractions being obtained which appear homogeneous on paper chromatograms. This approach is at present being investigated further with larger amounts of crude pigment, hoping to obtain sufficient material for elementary analysis and study of chemical structure and physical properties.

Significance of Research to the Institute: As the first representatives of a new group of natural compounds, the pigments from Elsinoe have general biochemical importance as has their photosensitizing (phototoxic) action. Such action has been established so far only for a small number of natural pigments. The various species of Elsinoe might also offer good possibilities to investigate experimentally the biosynthetic pathways by which their pigments are formed, and to test plausible but hypothetical [illegible] which have been proposed for the biosynthesis of closely related groups of natural pigments.

Significance to Research of the Institute: In view of the medicinal importance of codeine and related substances, and of the long-standing interest of NIH in this subject, any contribution to a better understanding of their chemical behavior is worthwhile. In the present case, an old error recorded in the chemical literature has been corrected; in addition, an apparently new type of chemical reaction has been observed, which may be of interest beyond the case at hand. Compounds of this new type may perhaps be found in other instances of reduction by zinc and acid, and may so far have been overlooked in the complex mixture of products which is often obtained.

Proposed Course of Project: This investigation is completed and no further work on this topic is contemplated.

Subproject Title: (2) Photochemistry of alkaloids and phenanthrene derivatives.

Objectives: Investigation of the transformations of organic molecules by ultraviolet or visible light.

Methods Employed: The organic compounds were irradiated in solution, using natural sunlight or laboratory sources of ultraviolet radiation. The experiments with the latter sources were performed by Dr. Sharpless; methods and findings are described in more detail in his Annual Report. Isolation and characterization of the resulting compounds were attempted by the usual chemical methods.

Major Findings: (a) Irradiation of thebaine. The opium alkaloid thebaine contains a homocyclic conjugated diene system somewhat analogous to the one responsible for the photochemical reactivity of ergosterol. The photosensitivity of thebaine, anticipated for this reason, was actually found on irradiation with UV light. The intense absorption band at 2840 Å, known to result from approximately equal contributions of the diene system and the aromatic ring of thebaine, decreases to about 50% of its initial intensity on irradiation under anaerobic conditions, indicating disappearance of the diene chromophore. Isolation of the reaction product(s) in pure form is under way. (b) Irradiation of 9-bromophenanthrene. The photochemical dimerization of 9-substituted anthracene derivatives has been studied repeatedly, but the behavior of the analogous 9-substituted phenanthrenes on irradiation has been given little attention. Dr. Ziffer has found that exposure to sunlight of a benzene solution of 9-bromophenanthrene results in its conversion into a crystalline compound, which differs from the parent substance by its high melting point and low solubility in organic solvents. Its chemical nature is under investigation.

Significance of Research to the Institute: Better understanding and more extensive knowledge of the transformations of organic molecules on irradiation is of fundamental importance for an understanding of light-induced biological processes. Although photochemical reactions have long been known to occur, interest in them was sporadic, and systematic investigation of the unusual structures often formed by such reactions has started only in recent years, so that knowledge on this area is far from sufficient.

Proposed Course of Project: Elucidation of the chemical structures of the phototransformation products of thebaine and 9-bromophenanthrene is planned. The investigation will be extended to other compounds which are sensitive to ultraviolet or visible light.

Subproject Title: (4) Optical Rotatory Dispersion with Dr. Elliot Charney.

Objectives: The objective of this research is the study of the optical rotatory dispersion of organic molecules having chromophoric groups other than carbonyl, to find out whether such an investigation is experimentally feasible, and, if so, whether its results are of value in organic-chemical and biological research.

Methods Employed: For the study of the wavelength dependence of optical activity throughout the visible and ultraviolet regions of the spectrum, the Rudolph Spectropolarimeter is generally used. This instrument requires manual setting of the wavelengths at which measurements are to be made. Recently, an accessory equipment has been introduced by Perkin-Elmer Company which makes it possible to route rotation of polarization through standard UV recording spectrophotometers. This equipment has been loaned to the Photobiology Laboratory for one week and has been tested for its ability to give information not readily obtained with other devices.

Major Findings: Study of the changes in optical rotation with wavelength has recently yielded results of great value in organic and biological chemistry. In particular, the anomalies of the rotatory dispersion occurring in the neighborhood of absorption bands in the ultraviolet have given much valuable information on chemical constitution, configuration and conformation of organic compounds. However, these studies have been mostly restricted to aldehydes and ketones. In those compounds, the absorption band causing the anomalous rotation is of low intensity, so that sufficient light is transmitted for polarimetric measurements at wavelengths fairly close to the absorption maximum. It would be desirable

Mr. Elton Chaney

to extend such studies to compounds with more intense bands, particularly to phenolic and polycyclic substances. Both groups include many products of great interest in chemistry, biochemistry, and pharmacology.

Proposed Course of Project: On the basis of our preliminary results, a further exploration of the optical rotatory dispersion of non-ketonic compounds seems worthwhile. The study might also include investigations of proteins, which have very high optical rotations caused by their helical conformations.

ACTIVITIES OTHER THAN RESEARCH

In January, 1959, I joined the Panel on Biochemistry and Nutrition of the Research Fellowships Review Branch, Division of Research Grants, NIH. In September, I was transferred to the newly founded Panel On Biophysical and Organic Chemistry.

At the request of the B and N Panel, I prepared a detailed memorandum on the question of NIH support for research and training in pure organic chemistry, coming to the conclusion that there was both need and justification for such support from NIH funds.

Part B included YES

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Bobbitt, J. M., Weiss, U., and Hamessian, D.: Anomalous Optical Rotatory Dispersion in the Morphine Series. Note in the J. Organic. Chem. 24: 1582, 1959.

1. Ph
2. Ba
3. Bethesda

FRS-NMB
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Effect of Nuclear Radiation on Biological Systems

Principal Investigator: Ernst Liddel

Other Investigators: Ellis S. Kempner, Charles W. Malich, Frank M. DeFilippes, (transferred to NIAID approximately 1 November 1959), E. L. Lewis, E. J. Holthaus

Cooperating Units: Department of Terrestrial Magnetism, Carnegie Institution

Man Years (calendar year 1959):
Total: 4-1/2
Professional: 3-1/2
Other: 1

Project Description:

Objectives: (1) An understanding of the effects of neutrons and γ-radiation on biological systems (a) by study of the molecular interactions which occur as a result of these radiations (b) by study of cytological changes which occur under radiation.

Methods Employed: Radiation of cultures of microorganisms and subsequent examination by microscope and other physical methods - e.g., induced radioactivity, spectroscopic analysis where feasible, including absorption and electron paramagnetic resonance.

Major Activities: A portion of the laboratory space assigned was made available for use in the late winter. A 50 curie Po-Be neutron source was installed in a graphite moderator in March and preliminary measurements made on the nature of the neutron flux. Biological studies included radiation of pneumococci cultures to see whether radiation had an effect on growth rate. The results were inconclusive because the neutron flux was too small

The second phase of laboratory rehabilitation scheduled for completion in March, was finally completed in December. This will enable the installation of a Van de Graaff generator which, with tritium targets, should provide a neutron flux at least 100 times greater than the Po source. This installation will provide the maximum source feasible in the space now available under the conditions of the space loan.

By use of a radioactive analog of an amino acid, phenylalanine, it has been shown that the final selection process in the synthesis of proteins of the yeast, Candida utilis, takes place in one of the two metabolic "pools" of the cell. This could be interpreted as an operational identification of a template mechanism of protein synthesis.

PHS - NIH
Individual Project Report
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Serial No. NIAMD-121
OADR
Mathematical Research
Bethesda, Maryland

Part A

Project Title: Mathematics of kinetics and reaction-transport systems.

Principal Investigator: John Z. Hearon

Man Years:

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

Objective: The chief objective is to conduct a systematic study of mathematical problems of complex reaction systems and systems in which chemical conversion and translocation simultaneously occur. The study is thus concerned with problems of rate behavior, transport, the relation between energetics and phenomenological rate equations in general and irreversible thermodynamics and chemical kinetics in particular. This project is strongly oriented towards actual problems of current interest in biology. However, there often arise problems of inherent mathematical interest and examples are cited, in what follows, which are of relevance to current mathematical research.

Major Results and Significance:

Matrix Theorems and Linear Analysis: It was reported last calendar year that it had been proved that the Jacobian Matrix for an arbitrary non-linear chemical system is necessarily similar to a symmetric matrix. Some of the consequences for rate behavior of metabolic systems and relaxation-time analysis were sketched. This theorem has been broadened so that it applies to certain diagonal stochastic matrices of current interest in random-walk problems. It has also been shown that the signs of the matrix elements can be uniquely assigned. This assignment is essential for the discussion of stability of equilibrium states in complex systems and the possible existence of multiple stationary states. These matters are of obvious importance in "triggering mechanisms" in physiological systems and in the dependence of stationary states upon initial states. Further progress of a practical nature has been made in terms of application to relaxation-time analyses. In particular it has been

shown that each conservation condition (or material balance) implies the existence of an eigen-vector corresponding to a zero root and that the totality of such vectors are linearly independent. Thus the general results are carried over to the reduced system of rate equations with which the experimenter usually deals. In addition to answering some general questions about rate behavior these results are of considerable interest in terms of the relation of thermodynamics to kinetics. For example, a large class of rate functions for autocatalysis are consistent with thermodynamic equilibrium results. But the symmetry requirement on the Jacobian matrix selects uniquely an admissable rate function. Some of these results were presented in an invited lecture before the American Mathematical Association.

Area Theorem: Also reported last year was a method for computing the area under a curve in terms of the coefficients of the differential equation of which that curve is a solution. Extensions of this theorem (prompted in part by collaborative work on clotting mechanism with Dr. R. N. Shulman, NIAMD) have been obtained: Special deductions from the general theorem enable certain results to be written down from the inspection of the reaction scheme. In particular by inspection it can be stated under what conditions the final yield of products will be independent of certain reaction steps in the scheme and independent of the initial conditions. Some aspects of the general theorem have been extended to non-linear systems and applied to the kinetics of prothrombin to thrombin conversion. One consequence of general chemical kinetic interest is that rate constants can be estimated from final yields in non-linear systems for which no analytical solutions for the rate equations are known.

Superposition Properties: In the clotting study referred to above it was found that inhibited and uninhibited curves of thrombin yield can be superimposed by multiplication by a scale factor as can uninhibited curves under different initial conditions. A mathematical study of these results showed the following: the superposition property for solutions of linear differential systems is well known. The problem here is the converse. It has been shown that superposition implies and is implied by homogeniety degree one of the differential system and the linear case is a special instance of this class. In terms of the clotting problem it results that the thrombin yield as a function of time, t, prothrombin, P, and inhibitor, I, is factorable into the product of three functions of these variables, e.g., $F(t)H(P)G(I)$. This result, independent of any specific kinetic scheme, used in conjunction with certain rather non-restrictive kinetic assumptions, affords independent kinetic evidence for the existence of an intermediate between prothrombin and thrombin, allows the irreversibility of certain steps to be established, and predicts

certain experimentally verified relation between uninhibited and inhibited yield-curves. There was posed by this analysis the following problem of some interest in matrix algebra: Given a matrix with elements a function of a variable x, under what conditions will the spectrum of the matrix consist of two subsets, one set independent of x? A set of sufficient conditions have been found. In application these conditions place immediate restrictions upon the kinetic scheme proposed.

Approximate Diffusion Equation: Work has continued on this as indicated in the 1958 report. The relation between the analytical and computational problems in this work was presented before an IBM Symposium on Computers in Biology and Medicine

Part B is included.

Part B:

Publications

Best, J. B. and Kearon, J. Z. Thermodynamics of Homeostasis. Chapter in Mineral Metabolism, edited by Bronner and Comar. New York, Academic Press, 1959.

Stetten, DeWitt, Jr. and Kearon, J. Z. Intellectual Level Measured by ACB and Serum Uric Acid Concentration. Science 129, 1737 (1959).

Arnoff, S. and Kearon, J. Z. Kinetic Models of Ascitase Action. In press. Archives of Biochem. and Biophys.

Kearon, J. Z. Consideration of Approximate Solutions of the Equation of Continuity. Accepted for publication in special IBM Symposium on Computers in Biology and Medicine.

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD-122
OADR
Mathematical Research
Bethesda, Maryland

Part A.

Project Title: Mathematical formulation and analysis of problems relevant to experimental neurophysiology

Principal Investigator: Wilfrid Rall

Man years:

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

Project Description:

Objectives:

(1) To develop explicit mathematical formulations of various neurophysiological hypotheses.

(2) To elaborate theoretical predictions that are well suited for experimental testing.

(3) To analyse and reassess certain current neurophysiological concepts involving simultaneous consideration of three different kinds of information (a) neuroanatomical data, (b) electrophysiological data, as well as (c) a mathematical formulation of their interdependence.

(4) To contribute to the interpretation and the design of some of the experiments conducted by neurophysiological colleagues at NIH.

Methods employed: The neurophysiological problem must be reduced to its essentials and then be formulated mathematically. Typically, this formulation is a partial differential equation that must be solved for a variety of boundary and initial conditions. The use of Laplace transforms has been very fruitful in solving several current problems.

Major Findings and Significance:

(1) The spread of electric current from a neuron soma into branching dendritic trees has been formulated mathematically.

Because of its importance to the interpretation of recent experiments performed upon motoneurons with intra-cellular micro-electrodes, considerable care has been devoted to the preparation of a paper (Experimental Neurology, 1959) which includes a careful assessment of assumptions, derivation of theory, practical formulae, and detailed application to the best experimental data currently available from anatomical and electrophysiological sources. On the basis of the current data, the results indicate that motoneuron dendrites play a dominant role (rather than the subsidiary role assumed by others) in determining important motoneuron properties. This has important implications for current concepts of synaptic excitation and reflex integration.

(2) Significant gains have been made on the more general problem of membrane potential spread over neuron soma and dendritic surfaces in response to synaptic current generation over these surfaces. In addition to numerous useful special cases, the solution to a very general problem has been obtained: the synaptic generator current can have an arbitrary time course, its intensity can have any one of a large variety of distributions over the soma and dendrites, and the initial condition of the membrane surface need not be the resting condition. Several difficulties and ambiguities in the theory of synaptic excitation and in the interpretation of recent experiments are now being analysed in terms of these theoretical results. This theory predicts, for example, the differences to be expected between a synaptic potential generated predominantly in the dendrites and one generated predominantly near the soma. A publication is in preparation; further significant applications are anticipated.

(3) Mr. Ezra Shahn and I have developed a procedure that will enable us to use the IBM 650 computer in the study of several questions of relevance to the extracellular electric potentials recorded by neurophysiologists. This procedure provides us with the electric potential field to be expected for various distributions of point current generators on the surface of a sphere. Most of the difficulties have now been overcome. Application of the results to neurophysiological problems remains to be done.

(4) Collaboration is in progress with Drs. K. Frank and P. G. Nelson, NINDB, in the design and interpretation of experiments with single motoneurons of cat spinal cord.

Proposed Course: Current results have raised further questions. Work will continue along the same general lines.

Part B

Publications:

Rall, W.: Branching dendritic trees and motoneuron membrane resistivity. Experimental Neurology 1: 491-527, 1959

Invited Lectures:

December 29, 1959 in a symposium entitled: "Mathematical Models in the Life Sciences" sponsored by The American Statistical Association, The Biometric Society, and The Institute of Mathematical Statistics.

November 25, 1959 for The Faculty Seminar of Southwestern Medical School, Dallas, Texas.

March 4, 1959 for the Neurophysiology and Neuroanatomy Seminar of the Walter Reed Army Medical Center

March 5, 1959 for the Physiology and Pharmacology Seminar of the George Washington University.

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Serial No. NIAMD-123 b
CADR
Mathematical Research
Bethesda, Maryland

Part A.

Project Title: Study of iodine kinetics in the thyroid system and radioiodine treatment of thyroid abnormalities.

Principal Investigator: Manes Berman

Other Investigators: Charles Lewallen
David Becker
Richard Benua
Martin Sonenberg

Cooperating Units: Clinical Endocrinology Branch, NIAMD, Serial No.
New York Hospital, New York
Sloan Kettering Institute, New York

Man Years:

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

Project Description:

Objective: The objective of this project is to develop a general model for the kinetics of iodine in the thyroid system that will explain the various thyroid disorders found in patients and that will agree with the various experimental data collected on these patients. It is also the purpose of this project to study the sites of action of radioiodine when used for therapy and the effect on iodine turn-over rates produced by radiation, drugs and hypophysectomy.

Methods employed: Patients having various thyroid abnormalities have been studied. Kinetic studies using radioiodine have been made on these patients over periods up to about 2 years. These include studies before and after treatment of the patients. The kinetic studies involve measurements of radioactivity in blood PBI, thyroid, urine at various times after a tracer amount is administered to the patient. From the collected data calculations are made of the turn-over rate of iodine from iodide to organically bound iodine in the thyroid, the secretion and degradation rates of thyroid hormone and other variables.

Application of mathematical models and computers to the analysis of the data is made in an effort to demonstrate if the models used are consistent with the data and to derive measures of uncertainty for the constants of the models. The methodology for this type of analysis is still being developed and the final analysis of the data is still pending.

The abnormalities studied are due mostly to hyperthyroidism and cancer. Effects produced by radiation treatment (I^{131} and I^{133}) hypophysectomy, thyroid and pituitary hormones have been studied.

Major Findings: The findings reported on last year have been found to apply to additional patients who underwent hypophysectomy. It was found that in some patients two populations of protein bound iodine must exist in order to explain the kinetic data. The populations have not been identified.

Significance of program to the Institute: Since the thyroid is a most important organ in the regulation of physiological processes, its detailed modes of action are of interest for understanding the thyroid as well as other metabolic systems in the body. Furthermore, the development of an analytical procedure to treat the homeostatic mechanism of the thyroid may also be applicable to other homeostatic mechanisms in the body.

Proposed course of project: The complete analysis of the data collected over the last few years is still pending the completion of the development of the methodology. When this is done, the analysis outlined earlier will be done.

New experiments are being planned to investigate two abnormalities found in the collected data. This will be done in collaboration with Drs. C. G. Lewallen and J. E. Rall of the Clinical Endocrinology Branch of the NIAMD.

Part B not included

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. NIAMD-123
OADR
Mathematical Research
Bethesda, Maryland

Part A

Project Title: Analysis of Radioisotope Tracer Data

Principal Investigator: Mones Berman

Other investigators: Ezra Shahn

Cooperating Units: Clinical Endocrinology Branch, NIAMD
Serial No. 147C

Man Years:

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

Project Description:

Objective: The objective of this project is to develop a mathematical and computational methodology for the systematic and routine analysis and interpretation of tracer data on steady state biological systems. The methods under development are intended to provide a rationale for how to choose a physiological model for a set of data and how to treat the data for the model chosen. It is also intended to program the procedures for routine use on analog and/or digital computers, and make them available to other investigators.

Methods employed:

1) Mathematical Theory: The development of a theoretical basis for the procedures of analysis to be used. This includes theory for model construction and data fitting.

2) Digital computer - programs have been written for fitting experimental data to models using high speed computers. The programs are being developed to be applicable to a variety of models and sufficiently flexible to take the experimental data directly.

3) Analog computer - The application of the analog computer has not been as extensive as anticipated in view of the success in using the digital computer. It was still used, however, for special problems.

Major Findings:

1) A comprehensive program to do a least squares fit of model constants to various forms of experimental data and general enough for a wide range of models has been written, and was applied to special problems. Further development and testing of the program is still in progress.

2) A method for obtaining the uncertainties of the model parameters that will take into account non-linearities of the system behavior is being tested.

3) Applications - The methods developed have been applied to problems of several investigators. These include

a) Glucose metabolism - Analyses of C^{14} labeled glucose kinetics data obtained by Dr. S. Segal of the Clinical Endocrinology Division have been made. These analyses brought out inconsistencies in proposed models, inadequacies in the collected data and suggested additional experimentation to justify new model proposed.

b) Initial application of the methods to iodine kinetics data on a series of patients collected by this investigator are in progress.

c) Assistance was given to a number of investigators in formulating their problems mathematically, and in the analysis of their data.

Significance to the Program of the Institute: A great deal of work is carried on using isotope tracer techniques. Interpretation of the collected data is usually made by postulating some compartmental model and solving for turn-over rates and compartment sizes of the model. It is assumed that the values obtained may either reflect mechanisms of action or indicate the sites of normal and abnormal processes. Such an analysis of data is limited at present to very simple systems because of the complexity in the analysis of multicompartmental systems. It is hoped that the methods developed here will enable investigators to study more complex systems, extract more information from their data and have a measure of confidence for the models they propose.

Proposed course of Project: The development of mathematics for rigorous procedures in formulating biological models and in analyzing data will continue. General computer programs applicable to a variety of problems will be developed. Application of developed methods will be pursued.

Part B included.

Part B

Publications:

Berman, M., Schoenfeld, R.: Information content of tracer data with respect to steady state systems. Symposium on Information Theory in Biology, H. P. Yockey, editor, Pgs. 181-6, (1958)

Lewallen, C. G., Berman, M. and Rall, J. E.: A mathematical approach to the kinetics. J. Clin. Invest. 38: 66-87, Jan. 1959.

Lewallen, C. G., Rall, J. E. and Berman, M.: Studies of Iodo-albumin Metabolism. II. The effects of thyroid hormone. J. Clin. Invest. 38, 88-101, Jan. 1959.

Berman, M. and Schoenfeld, R.: A note on unique models in tracer kinetics. J. Exper. Cell. Research. In press.

Serial No. NIAMD-124C
1. Clinical Investigations
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Trial of New Anti-Rheumatic Drugs

Principal Investigator: Dr. Joseph J. Bunim

Other Investigators: Drs. Roger L. Block, Kurt J. Bloch, George E. Ehrlich, Nathan J. Zvaifler, and Alexander Deutsch

Cooperating Units: The Georgetown Medical Service of the D.C. General Hospital

Man Years (calendar year 1959):
Total: 1-5/6
Professional: 1-5/6
Other: 0

Project Description:

Objectives:

The objectives of this project remain as in previous years:

a) To determine the relative anti-inflammatory potency in man.

b) To detect and report early the undesirable side effects, if any.

c) To delineate families of compounds showing most promise as therapeutic agents.

Methods Employed:

Evaluation of therapeutic compounds has been conducted with in-patient and on out-patient populations. The in-patient group has been observed at the Clinical Center.

Part B included Yes [X] No [ ]

The out-patients have been studied in the Arthritis Clinic of the Rheumatic Disease Service at D.C. General Hospital. This Clinic was established through the cooperation of the National Institute of Arthritis and Metabolic Diseases and the Georgetown Medical Service. Patients under study are observed carefully during a control period and during the period of test drug administration. Anti-inflammatory indices, laboratory evaluation, and metabolic and hormonal studies have been utilized. Details of the studies have been published.

Patient Material:

Major Findings:

The following compounds have been studied during 1959:

1) Dexamethasone (16α methyl 9α fluoroprednisolone)
2) Isoxicam (a condensation product of isoniazide and m-benzaldehyde sulfuric acid)
3) (6α fluoro triamcinolone)
4) (6α fluoroprednisolone)

The dexamethasone study was extended during the previous year to include a total of 27 patients with rheumatoid arthritis. These patients have now been observed for up to 21 months, while receiving from 0.5 to 4.0 mg. of dexamethasone daily. Salicylate (acetylsalicylic acid or buffered A.S.A.) in doses of 2.4 to 6.0 gm. daily was added to the regimen of 13 of the 27 patients with the result that maintenance dexamethasone dose could be lowered an average of 1.5 mg. daily in this group. (Three were unable to decrease the maintenance dose). In the non-salicylate group, the maintenance dose was lowered in only 2 (0.3 and 0.5 mg. respectively). The functional improvement in this group is shown as follows:

	Previous Regimen	On Dexamethasone			
		Class			
Functional Class	Number of Pts.	1	2	3	4
1. No impairment	0	-	-	-	-
2. Some impairment	10	9	1	-	-
3. Marked impairment	11	2	6	3	-
4. Confined to bed or chair	4	1	1	2	-

Studies of the effects upon glucose metabolism were continued this year. Five patients showed inhibition of glucose utilization rate (GUR) during dexamethasone administration. Five others showed improvement in GUR while the remainder of the patients remained the same as when first observed during the control period.

The effect of dexamethasone upon parameters of thyroid function was also studied. Although control values were not available for periods of non-corticosteroid therapy, there was a correlation between magnitude of the dexamethasone dose and the depression of the radioactive iodine uptake (RAI) and protein bound iodine (PBI) values. Basal metabolic rate (BMR) and serum cholesterol values showed no such correlation. While receiving continued maintenance dexamethasone the three patients with the most abnormal RAI and PBI values were given thyroid stimulating hormone (TSH), 10 units daily for three days. In two patients the RAI, PBI and BMR values returned to normal. The third patient, who did not respond, revealed in her serum high titers of antibodies against thyroglobulin. This evidence, although consistent, did not prove a suppressive effect by dexamethasone upon TSH production.

The most prevalent side effects were facial rounding in 25 and appetite increase in 23 of 27 patients, with weight gain of over 4 kg. in 19 cases. Petechiae or easy bruising were noted in 11 patients, and 8 had edema. Epigastric pain occurred in 4, one developed a duodenal ulcer and one other experienced an exacerbation of an old duodenal ulcer. Three patients developed pathologic fractures. One patient developed hypertension. Four patients died during the study period; one, a 57-year-old man, died with thrombophlebitis and pericarditis; another, with a lung abscess, died during a period of dexamethasone withdrawal (from 3.5 to 1.5 mg. daily) with added ACTH and DOCA; a third, a 37-year-old female, died during surgery with acute pancreatitis; and the fourth, a 45-year-old female, died during surgery for intestinal obstruction.

Isorilone, a condensation product of isonicazide and m-benzaldehyde sulfuric acid, was found to have anti-inflammatory properties in animal studies. This compound was administered to two patients with rheumatoid arthritis who showed some decrease in joint swelling and tenderness while receiving 6 gm. daily. A triple blind study with four other patients was then begun. Periods of placebo, aspirin, 3.6 gm. daily, and isorilone, 6.0 gm. daily, were instituted and alternated without the knowledge of the patient or observer. The latter performed careful evaluations of degree of articular inflammation every 3 days. At the end of the study it was found that the period of greatest rheumatic activity in all 4 cases corresponded to the period of isorilone administration. No serious side effects were observed during the period of the study.

6α fluoro triamcinolone, synthesized by Dr. J. Fried of Squibb and Company, has been administered to 5 rheumatoid arthritis patients, four in a short term evaluation and 1 on a metabolic study. The anti-inflammatory potency was carefully titrated in 4 patients and found to be one-fourth to one-third that of dexamethasone and two to three times that of triamcinolone. During the 6 to 60 day period of these short term trials, no serious side effects were encountered. Doses of 3 to 10 mg. were employed. One other patient participated in a metabolic study, receiving 20 mg. daily. No remarkable alterations of sodium or potassium balance were observed. The calcium balance tended to be positive, but data was not conclusive. Additional studies with this compound are anticipated.

6α fluoroprednisolone, synthesized by chemists of Upjohn Company, has been administered to three patients with rheumatoid arthritis. Its anti-inflammatory effect it was found to be the equivalent of one-third to one-half that of dexamethasone. The doses employed varied from 5 to 10 mg. daily. 6α fluoroprednisolone had to be discontinued in one case (10 mg. daily) when the patient developed a gastric ulcer four months after the start of therapy. One other patient (7 mg. daily) developed severe Cushingoid facies with heavy fat deposits in the face, neck and abdomen. 6α fluoroprednisolone was discontinued and the patient reported less dyspnea, a symptom previously

causing marked dizziness. A third patient (8 mg. daily) has shown excellent control of symptoms for 8 months, without remarkable side effects.

Significance to NIAMD Research:

As in previous years, this study continues to provide experience with new therapeutic compounds and promotes improved management of rheumatic disease patients. It has also provided leads for further investigation in the basic mechanism of corticosteroid action.

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3) Smyth, C. J., Bunim, J. J., Clark, W. S., Crain, D. C., Demartini, F. E., Duff, I. F., Englemann, E. P., Graham, D. C., Montgomery, M. M., Norcross, B. M., Polley, H. F., Ropes, M. W., and Rosenberg, E. F.: Rheumatism and Arthritis - Review of American and English literature of recent years (XII Rheumatism Review), Part I, Ann.Intern.Med., 50:366-494, 1959. Part II, Ann.Intern.Med., 50:634-801, 1959.

4) Perence, C., Markowitz, M., and Bunim, J. J.: The effect of large doses of prednisone on acute rheumatic fever: Observations on the treatment of 17 patients with carditis with a 2-year follow-up. A.M.A. J.Dis.Child., 97:561-570, 1959.

5) Rogers, D. M., and Black, R. L.: Medical Interview: Rheumatoid arthritis. Gen.Practit. 19:102-108, 1959.

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7) Bunim, J. J.: Corticosteroids: Chemistry, physiology, and metabolic effects, in Arthritis, Hollander, J. L. (ed.), Philadelphia, Lea and Febiger, 1960, ed. 6, in press.

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9) Bunim, J. J.: Sarcoidosis and sarcoid arthritis. Ibid.

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11) Bunim, J. J.: Recognition and treatment of arthritis in the elderly patient. So.Med.Asso.Jl., 1960, in press.

12) Peterson, R. E., Black, R. L., and Bunim, J. J.: Disposition of intra-articularly injected cortisone and hydrocortisone. Arth.& Rheum. 2:433-439, 1959.

13) Peterson, R. E., Mokas, G., and Black, R. L.: Estrogens and adrenocortical function in man. J.Clin.Endocrin.& Metab., in press.

14) Black, R. L.: Survey of recent research activities in the rheumatic diseases. Arch.of Phys.Med.& Rehab., in press.

NIH-NIH

Individual Project Report
Calendar Year 1959

Part A.

Project Title: The Bentonite Flocculation Test in Rheumatoid Arthritis

Principal Investigator: Dr. Kurt J. Bloch

Other Investigators: Dr. Joseph J. Bunim

Cooperating Units: Drs. Edwin Lerner II (LPH 8R), E. Lemone Yielding, Thomas A. Burch, and Mr. Harold Neu

Man Years (calendar year 1959):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives:

To continue the evaluation of the bentonite flocculation test (BFT) as a diagnostic aid in rheumatoid arthritis.

Methods Employed; Major Findings:

Sensitivity and Specificity of the Bentonite Flocculation Test.

Evaluation of the sensitivity and specificity of the BFT as a serologic tool in rheumatoid arthritis has been continued in the past year. A total of 1500 tests have been performed on in-patients, and followup patients, and other referrals. A total of 200 patients with definite rheumatoid arthritis have been tested; of these 175 were positive and 25 were negative (approximately 90% positive). Twenty-seven patients with juvenile onset of rheumatoid arthritis have been tested; of these 3 (11%)

Part B included Yes [X] No [ ]

had a positive test. Although juvenile rheumatoid arthritis is considered to be the childhood counterpart of the adult disease, serological tests generally tend to be positive in only 10% of these patients. The collagen diseases continued to contribute the next highest incidence of positive test. In systemic lupus erythematosus 10 of 18 patients had positive tests; in scleroderma 6 of 13 patients had positive tests. Among patients with non-rheumatoid arthritides the following results were obtained: thirteen patients with osteoarthritis had negative tests; eight patients with ankylosing spondylitis had negative tests. A total of 67 patients with gout and/or hyperuricemia were tested; of these 4 had a positive test. One of these patients belongs to a family in which many members have a positive RFT in the absence of joint disease; two other patients probably have rheumatoid arthritis and gout; in the fourth patient the positive RFT may be due to abnormal proteins produced by a diseased liver. Thirteen patients with psoriasis and arthritis were tested; of these only one had a positive test, although several patients had a form of arthritis clinically indistinguishable from rheumatoid arthritis. One of 15 patients with Reiter's syndrome had a positive test.

Observations on the Relationship between Clinical Course of Rheumatoid Arthritis and Serological Titer.

The RFT has been performed, at weekly intervals, on all rheumatoid patients admitted to our Unit. The clinical course and serological titer of 20 patients have been correlated. There did not appear to be any significant change in RFT titer during remissions or exacerbations of the disease. Several patients had remarkably constant titers over several years. There tended to be a correlation between advanced stages of the disease process and high RFT titers.

Use of the RFT in Epidemiological Screening.

The RFT was performed on several hundred Eskimo sera collected during an epidemiological study in Alaska. It was found that the incidence of positive reactions in apparently healthy natives was similar to that found in the United States populations studied: approximately 2-3%. Sera from 239 inhabitants of the Marshall Islands were also tested and of these 5 (2.1%) were positive.

Four hundred and eighty-nine sera obtained during an epidemiological study in Wensleydale, England, were tested. These sera have also been tested by Dr. Ball, in England, using the sensitized sheep cell test. Eventually the results of this study will be correlated with the clinical and x-ray data obtained on this population.

The RFT was also used to test sera obtained by a United States Public Health Service Health Survey Unit in its various studies.

The Capillary Tube Latex Fixation Test (C.L.F.T.) and Its Application to Serological Investigation among Family Members of Rheumatoid Patients.

Several investigators have reported a significantly greater incidence of positive serological tests for rheumatoid arthritis in family members of rheumatoid patients than among matched control groups. The capillary tube latex fixation test employing heat aggregated human gamma globulin apparently detects "rheumatoid factor" among relatives of rheumatoid patients.

Mr. Harold Neu, a CO-OPHP student during the summer of 1959, experimented with this procedure under our supervision. It was found that a 1:5 dilution of serum in glycine saline pH 8.2, followed by inactivation at 56° C for 30 minutes, provided optimum conditions for testing serum. To this was added a latex suspension (diluted so that 0.1 ml. of latex in 10 ml. of buffer gave 20% light transmission in a Coleman Junior Spectrophotometer) mixed with an equal volume of heated gamma globulin solution. The mixture of diluted serum and gamma globulin coated latex was drawn into a capillary tube and allowed to stand at room temperature for one hour. At this time it was observed for agglutination. Seventy of 78 patients with classical or definite rheumatoid arthritis had a positive C.L.F.T.; six of 10 patients with probable or possible rheumatoid arthritis were also positive. There were 3 positive tests in 72 patients with non-rheumatoid arthritis. Five of 191 employees, 2 of 109 blood donors and none of 22 normal volunteers were positive.

Comparison of the results obtained in the C.L.F.T. and BFT revealed agreement in 76 of 79 patients with classical or definite rheumatoid arthritis and in 14 of 18 cases of probable or possible rheumatoid arthritis. The C.L.F.T. appeared to be slightly more sensitive than the BFT in detecting rheumatoid factor in patients with rheumatoid arthritis but this was accompanied by a slight loss of specificity.

Previous experiments using the BFT had indicated that relatives of rheumatoid arthritic patients did not have an unusual incidence of positive tests by this procedure. However, with C.L.F.T., 7 of 64 relatives of rheumatoid patients (11%) and 4 of 33 relatives of patients with juvenile rheumatoid arthritis (12%) had positive tests. One of 92 relatives from a control group had a positive test (1%). These results suggest that the presence of rheumatoid factor in serum may be genetically determined.

The BFT in Experimental Arthritis.

It was previously reported that rats injected with live Streptobacillus moniliformis developed arthritis accompanied by positive serological tests for rheumatoid arthritis. These serological results were also produced in rats as a response to killed antigens which did not produce joint lesions. Immunisation of rabbits with killed antigens produced high BFT titers. The factor in rabbit serum responsible for the BFT reaction was shown by immunological and physical methods to be distinct from human rheumatoid factor and appeared to be an antibody to human proteins. Rats injected with Streptobacillus moniliformis grown in the absence of human proteins developed joint lesions but no elevated BFT titers. The presence of human protein in the culture medium used for this microorganism appeared essential for the production of elevated BFT titers in the sera of injected or immunized rats.

Effect of Penicillamine on Rheumatoid Factor as Measured in the BFT.

Rheumatoid factor is a macroglobulin which appears to consist of smaller proteins linked by disulfide bridges. Treatment of rheumatoid sera with sulfhydryl compounds leads to complete loss of serologic activity.

Reports from several investigators suggested that penicillamine was able to disrupt the macroglobulins of Waldenström's macroglobulinemia, and that this was associated with some improvement in the patient's course. It was therefore decided to test the effect of penicillamine on rheumatoid factor both in vitro and in vivo. Experiments by Dr. E. Leanne Yielding and Mrs. Louise Yielding indicated that treatment of rheumatoid serum with relatively high concentrations of penicillamine lead to loss of serologic activity in the RFT. Penicillamine was given to two patients, one with rheumatoid arthritis and one with scleroderma, both of whom had high RFT titers. A one week trial of 750 mg. penicillamine daily failed to affect the RFT titer in either patient. A further course of 1.5 gm. of penicillamine daily was given to the patient with scleroderma. Again there was no change in RFT titer; slowly decreasing total leukocyte counts were observed and the experiment discontinued. Penicillamine did not affect the titer of another serum macroglobulin, i.e., isoagglutinin, nor did it effectively remove calcium from the patient with scleroderma and calcinosis cutis.

Significance to NIAMD Research:

The RFT is an important tool for the clinical and experimental investigation of serological reactions in rheumatoid arthritis.

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Individual Project Report
Calendar Year 1959

Part B.

Honors, Awards, and Publications

Publications other than abstracts from this project:

1) Bloch, K. J., and Dunin, J. J.: Simple, rapid diagnostic test for rheumatoid arthritis---bentonite flocculation test. J.A.M.A. 169:307-314, 1959.

2) Bloch, K. J.: Recent modifications in serological tests for rheumatoid arthritis. Bull.Rheum.Dis. 9:185-188, 1959.

3) Lerner, E. M., II, Bloch, K. J., and Williams, R. R.: "Rheumatoid"serological reactions in experimental animals - the sensitized sheep cell hemagglutination reaction and bentonite flocculation test in rats with experimental arthritis. (TO BE PUBLISHED IN ARTHRITIS AND RHEUMATISM.)

1. Clinical Investigation
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Sjögren's Syndrome

Principal Investigator: Dr. Kurt J. Bloch

Other Investigators: Drs. Martin J. Wohl, Irwin Shiy (NIDR), Robert Stephan (NIDR), Richard Oglesby (NIDR), Joseph J. Bunim, William R. Carroll, Richard A. Malmgren (NCI), Sheldon Kahn (NIH), and Philip R. McMaster (NIAID)

Cooperating Units: NIDR, NINDB, NCI, NIAID, and NIH

Man Years (calendar year 1959):

Total: 1-2/3
Professional: 1-2/3
Other: 0

Project Description:

Objective:

To study the clinical, serological, immunological and pathological spectrum of Sjogren's syndrome and its relationship to rheumatoid arthritis and other connective tissue diseases.

Methods Employed: Major Findings:

Clinical Aspects.

Twenty-one female patients with clinical evidence of Sjogren's syndrome were studied until the present time. The diagnosis of Sjögren's syndrome was based on symptoms related to dryness of mucous membranes and symptoms of the related connective tissue disease. Confirmatory examinations and laboratory studies were performed by cooperating units in the Ophthalmology Service (Dr. Richard Oglesby) and NIDR.

Part B included Yes [ ] No [X]

The diagnosis of keratoconjunctivitis sicca was based on filamentary keratitis demonstrated by biomicroscopy, decreased tear flow demonstrated by Schirmer Test #1, and abnormal staining of the bulbar conjunctiva and cornea by Bengal-rose stain. Dental examination disclosed dryness of the oral mucous membranes, unusual pattern of dental caries in some patients and enlargement of major salivary glands. Parotid flow was measured by use of the Lashley cup and indicated marked reduction or complete absence of salivary flow in several patients in this group (Dr. Robert Stephan). On secretory sialography of the parotid gland, sialectasis was demonstrated in all of the patients examined (Dr. Irwin Ship). Approximately 40 other patients have been examined by these techniques and will serve as a control group. According to the American Rheumatism Association criteria for the diagnosis of rheumatoid arthritis the initial group of 21 patients was divided into four groups. In the first group were 8 patients with definite or classical rheumatoid arthritis; in the second group were 3 patients with possible rheumatoid arthritis; two had scleroderma and 8 patients had only oral and ocular manifestations of Sjogren's syndrome.

Laboratory Examinations.

An unusual finding in this group of patients was a combination of low fixed urinary specific gravity and low blood urea nitrogen. This may be related to the chronic intake of large amounts of water. (This phase of our study is being conducted by Dr. Sheldon Kahn - NHI). Six of these patients had low white blood cell counts and many had eosinophilia. Two patients had thrombocytopenia without purpura. The erythrocyte sedimentation rate was elevated in 20 of 21 patients.

Serum Protein Changes.

Total serum proteins were less than normal in the first 3 groups of patients and were increased in the patients in group 4. Concentration of serum albumin was below normal in the entire group. Serum globulin concentration was increased in all 4 groups; a marked increase was noted in the gamma globulin concentration, especially in the last group.

Serological Reactions.

The bentonite flocculation test (BFT) was positive in 20 of 21 patients and the sensitized sheep cell agglutination test was positive in 19 of 20 tested. The serum component responsible for the positive BFT was located in the bottom fraction or pellet obtained by ultracentrifugation through a sucrose density gradient. Sera from the 4 groups behaved similarly. The serologically active fractions contained less than 10% of the total protein used in each experiment and contained all of the high molecular weight gamma globulin (19S) as determined immunologically by gel diffusion using an antiserum to 19S gamma globulin.

Analytical ultracentrifugation (Dr. William R. Carroll) revealed unusual high molecular weight complexes with a sedimentation constant of approximately 22, only in sera from patients with Sjogren's syndrome and rheumatoid arthritis. (These 22S complexes have been described in approximately 30% of rheumatoid arthritis patients, especially those with very high titers in serological tests for rheumatoid arthritis). Furthermore, these ultracentrifugation studies indicated that the increase in gamma globulin concentration was due chiefly to an increase in the 7S class of proteins.

Serum constituents with special affinity for tissue nuclei were demonstrated using fluorescent antihuman gamma globulin (Dr. Richard A. Malmgren). Significant titers were obtained with 13 of 21 sera from patients in this study. In comparison, only 4 of 27 rheumatoid patients without Sjogren's syndrome had significant titers in this technique. However, only 2 patients had positive L.E. cell preparations; in one of these patients this was associated with clinical evidence of systemic lupus erythematosus.

Five of the patients in this group had positive direct Coomb's tests. Studies are proceeding to identify the antibody coating red blood cells in these patients. Three of the patients had antibodies to thyroglobulin coated, tannic acid treated sheep erythrocytes. There was no evidence of clinical thyroid disease in these patients.

Experimental Studies.

Attempts to demonstrate antibodies to saline extracts of human salivary gland have been inconclusive. Preliminary experiments using complement fixation indicated that sera of patients with Sjögren's syndrome reacted with saline extracts and fixed complement. These studies are to be further confirmed and extended.

Experimental Production of Sjögren's Syndrome.

Experiments have been started in guinea pigs injected with homogenates of lacrimal glands emulsified in Freund's adjuvant. After five weeks these animals showed skin reactivity to homogenates of lacrimal gland. Histologic examination of tissue from these animals is pending.

Significance to NIAMD Research:

Sjögren's syndrome is a little known variant of rheumatoid arthritis. Preliminary studies suggest that it may be due to an autoimmune process which interferes with the function of glands responsible for moistening various mucous membranes. A better understanding of this syndrome may increase our knowledge of rheumatoid arthritis and related diseases.

Future Experiments.

1) Further attempts will be made to demonstrate antibodies to salivary and lacrimal gland constituents using complement fixation, precipitation, and agglutination techniques.

2) Clinical studies will continue in order to define the full spectrum of this syndrome, including study of patients with only oral or ocular disease.

3) The renal defect in Sjögren's syndrome will be further defined.

4) Studies will continue to delineate the pattern of dental caries and abrasion seen in patients with Sjögren's syndrome.

5) Studies are planned to investigate the clinical and serological manifestations among the family members with this syndrome.

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1. Clinical Investigations
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Genetic Polymorphisms in Man and Other Animals

Principal Investigator: Dr. Baruch S. Blumberg

Other Investigators: Drs. Jacob Robbins and J. Edward Hall; Mr. Laurence Forer; Dr. Stanley Gartler, University of Washington, Seattle; and Dr. Hugh Fudenberg, Rockefeller Institute, New York, New York

Cooperating Units: There were no cooperating units elsewhere in the Public Health Service

Man Years (calendar year 1959):
Total: 2-1/4
Professional: 1-1/4
Other: 1

Project Description:

Objective:

To determine the presence of genetic biochemical polymorphisms in man and other animals, to investigate their distribution in different populations, and to determine their relationship to the arthritides and other diseases.

Methods Employed; Major Findings:

General - Many biochemical traits in humans and other animals are genetically determined. Some of these may be classified as genetic polymorphisms; that is, the existence in a population of two or more easily distinguished forms of a trait, the lesser of which could not be maintained by recurrent mutations alone. The normal hemoglobin-sickle cell hemoglobin system, most of

Part B included Yes [X] No [ ]

the blood groups, and other systems to be described below, fall within this classification. From studies in lower animals and theoretical considerations, there is reason to believe that selection may operate to maintain these traits in the incidences found and in some cases the selective forces may be related to disease. It is of interest to determine if populations living under different environmental conditions and prone to different diseases have different incidences of these genes. In addition, studies of the distribution of these traits can sometimes provide information on genetic relations between separated population groups. A field trip to the Central Pacific was made in the winter of early 1959.

Haptoglobins.

These are a family of serum proteins which bind hemoglobin. There are three major patterns in humans and these are under genetic control. Some rare types have been discovered by an NIMH scientist and other workers. Bloods were collected from Micronesians living on the atolls of Rongelap, Utirik, and Majuro in the Marshall Islands. The prevalence of type 1-1 was found to be high in the Rongelap people, although not quite so elevated in the smaller number of cases studied from the other atolls. Several individuals with no haptoglobin were found; this would presumably be of physiological importance under some conditions of red blood cell breakdown. In two cases it was found that individuals who had no haptoglobin, in 1959, had small amounts of haptoglobin in 1957. This implies a phenotypic change during the course of the two years.

A case of paroxysmal cold hemoglobinuria was studied in conjunction with Dr. M. R. Shulman. It was found that the patient had no detectable haptoglobin following the acute hemolytic episode, and that the haptoglobin returned to nearly normal amounts after recovery. However, the haptoglobin dropped to undetectable levels later, apparently independent of the hemolytic crisis. This suggested that the haptoglobin-producing mechanism is independent of the blood loss.

Monkeys, chimpanzees and baboons were found to have only one of the haptoglobin types, and, presumably, one of the genes. This implies that the polymorphism originated in human populations and has been perpetuated by forces of selection present in humans but absent in lower primates. The haptoglobins have also been studied in a variety of animals and interesting species differences observed.

Gamma Globulin Groups.

The agglutination test for "rheumatoid factor" has been used as a diagnostic method for rheumatoid arthritis. It has recently been shown by Grubb that the sera of some humans will inhibit this reaction, and that the inhibiting material travels in the gamma globulin fraction. This inhibiting property is determined by an allelic pair of autosomal genes, Gm^a and Gm^b. A third gene, Gm^c, which may or may not be at the same locus, has also been detected by Steinberg. In a preliminary study of African, Eskimo, Alaskan Indian, and Micronesian populations, it has been shown that the Gm^b gene appears to be absent from these populations. Studies on the Gm^c gene are in progress. Furthermore, it was found that there is a striking variation in the gamma globulin levels in these populations. Thus, some apparently normal Africans have total gamma globulins three times that of normal white Americans. The significance of this finding in relation to immunity may be of importance. This work was done in collaboration with Dr. Hugh Fudenberg of the Rockefeller Institute.

Urinary G-Aminoisobutyric Acid (BAIB) Excretion.

It has been shown by Gartler and others that the excretion of BAIB is, in part, under genetic control. Some persons with leukemia and other cancers are also high excretors, but the genetic role in these cases is not clear. Individuals who excrete large amounts of BAIB are rare in European populations. Approximately 200 urine samples from Micronesians were studied. It was found that nearly 90% of these were high excretors as compared to 10% high excretors in white American populations. Some of the Micronesians studied had been

subjected to fallout in 1954 following the detonation of a nuclear device on nearby Bikini atoll. It has been shown that radiation can increase the urinary BAID output. However, it is unlikely that this is the explanation of the present findings; there was no difference between the exposed and unexposed groups, and there was a high prevalence of high excretors in a small Micronesian population, from a nearby atoll with nearly normal levels of radiation. An alternate explanation is that a focus of the high excretor genes is present in Oceania or Southeast Asia. Studies to determine if this is so are in progress.

Taste Test.

The ability to taste phenylthiocarbimide (PTC) and related compounds in certain concentrations is genetically determined, although the threshold distinguishing tasters from non-tasters may vary in different populations. PTC and related substances are chemically similar to some goiterogenic materials and it has been suggested that there is an association between the taste-non-taste polymorphisms and thyroid disease. Preliminary studies have been completed to determine if the PTC taste dimorphism exists in experimental animals. It appears to be present in Macaca mulatta, the rhesus monkey.

Thyroxin Binding Proteins of Serum.

In conjunction with Drs. Jacob Robbins and J. Edward Rall, and Mr. Laurence Farer, the serum proteins which bind thyroxin have been studied. Four separate binding bands have been detected, representing a much more complicated pattern than had been suspected. These bands have been correlated with those seen on paper electrophoresis, by the use of two dimensional paper-paper and paper-gel electrophoresis studies. Variations in the patterns in various disease conditions have been studied, and some significant alterations noted. Species differences in binding patterns have also been found.

A variation in the position of the fastest moving band (the thyroxin binding pre-albumin) has been found in Macaca mulatta, and this may represent a polymorphism. In order to determine if this is so, studies on monkey families are contemplated. The relation of these variations in protein binding to differences in thyroid physiology could be the subject of further study.

Genetic Studies in Arthritis.

A review of studies on genetics and rheumatoid arthritis has been completed.

The Prevalence of Arthritis in an Eskimo Community.

Rheumatoid arthritis was found to be present in the Alaskan Eskimo community of Wainwright. A surprising finding was the low incidence of osteo-arthritis of hands and wrists in the Eskimo. This was found to be significantly lower than in an American white population corrected for age and sex. A high prevalence of individuals with positive bentonite flocculation tests was found in the village of Wainwright.

Proposed Course of Project:

1. A study of the biochemical and biophysical properties of the haptoglobins using specific enzymes for degradation studies.

2. A study of the antigenic relations of the haptoglobins, beta-globulin and gamma globulin types.

3. Further studies on the distribution of polymorphic traits in different populations and their relations to disease.

4. A statistical study of reproductive capacity in patients with rheumatoid spondylitis.

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Individual Project Report
Calendar Year 1959

Part B.

Honors, Awards and Publications

Publications other than abstracts from this project:

1) Blumberg, B. S., Ogston, A. G., Lowther, D. A., and Rogers, H. J.: Physicochemical properties of hyaluronic acid formed by streptococcus haemolyticus. Biochem.J. 70:1-4, 1958.

2) Blumberg, B. S., Allison, A. C., and Garry, B.: The haptoglobins, hemoglobins, and serum proteins of the Alaskan fur seal, ground squirrel, and mammot. J.Cell.Comp.Phys., in press.

3) Blumberg, B., Allison, A. C., and Garry, B.: The haptoglobins and hemoglobins of Alaskan Eskimos and Indians. Ann.Hum.Genet., in press.

4) Allison, A. C., and Blumberg, B. S.: Ability to taste phenylthiocarbamide among Alaskan Eskimos and other populations. Hum.Biol., in press.

5) Blumberg, B.: Genetics and rheumatoid arthritis. Arth.&Rheum., in press.

6) Corcoran, P., Alles, F. H., Allison, A. C., and Blumberg, B. S.: Blood groups of Alaskan Eskimos and Indians. Am.J.Phys.Anthrop., in press.

7) Allison, A. C., Blumberg, B. S., and Gartler, S. M.: Urinary excretion of β-aminoisobutyric acid in Eskimos and Indian populations of Alaska. Nature 183:118-119, 1959.

8) Blumberg, B. S., and Gartler, S. M.: High prevalence of high level PAH3 excretors in micronesians. Nature, in press.

9) Conard, R. A., Meyer, L. M., Suter, W. W., Blumberg, B. S., Lowery, A., Cohn, S., Lewis, W. H., Jr., Hollingsworth, J. W., and Lyon, H. W.: Medical status of Marshall Islanders in 1959 - five years after exposure to fallout radiation. Strahlentherapie, in press.

Honors and awards relating to this project:

Dr. Blumberg was named Assistant Editor of the journal, Arthritis and Rheumatism.

Serial No. [illegible]
1. Clinical Investigations
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: In Vitro Effects of Steroids

Principal Investigator: Dr. K. Lemone Yielding

Other Investigators: Drs. Gordon M. Tomkins and Alexander Deutsch

Cooperating Units: Dr. Laurence M. Gerwin (LMB 6)
Dr. Michael Potter (NCI) - 427a

Man Years (calendar year 1959):
Total: 2
Professional: 1
Other: 1

Project Description:

Objective:

To observe and explain in vitro steroid effects, ultimately attempting to define mechanism(s) of action at the molecular level.

Methods Employed:

Tissue homogenates, extracts, and purified enzymes were studied, using direct enzyme assays, isotopic tracer techniques, and specific chemical analyses. Animals were subjected to pre-treatment where indicated.

Major Findings:

DPNH Oxidation.

Low concentrations of various hormonally active steroids and stilbesterol inhibit DPNH oxidation (but not TPNH oxidation) by enzyme preparations from a number of sources (non-competitive with DPNH). The effect is catalytic

Part B included Yes [X] No [ ]

and not related to steroid metabolism. In addition to variations in sensitivity from organ to organ, there is a 1000-fold variation in range of potency among the active steroids. In kidney, the Ki (or half maximum inhibitory concentration) for stilbesterol is 8×10^{-7} M. This means that a measurable effect can be observed with concentrations as low as .02 gamma/cc. Cholesterol and tetrahydro E are ineffective. The effect has been studied in brain, spleen, muscle, heart, liver, thymus, and kidney of the rat. Beef pituitary is also under study.

Extension of studies to include preparations from E. coli, B. subtilis, and yeast (S. fragilis) revealed a similar inhibition of DPNH oxidation, and this could be correlated with known effects of steroids on growth. The effect was also demonstrated in preparations from Ehrlich and S-37 mouse ascites tumor.

Further refinement of studies has revealed the site of inhibition to be DPNH-cytochrome C reductase (specifically between flavoprotein and cytochrome b), a major link in the chain of hydrogen transfer in the cell's oxidative reactions. Interestingly, the inhibition is competitively reversed by tocopherol. This suggested need for investigation into a possible relationship between steroid and vitamin E. Examination of the tissues of vitamin E deficient rats, however, did not reveal differences in DPNH cytochrome C reductase or the degree of steroid inhibition*.

Further study of the effect has revealed interesting differences in response to added cytochrome C. In liver and kidney, the degree of steroid inhibition was sharply decreased by the addition of cytochrome C, while in heart and skeletal muscle, the steroid response was unaffected. Additional data now have confirmed the presence of an alternate route of electron transport between flavoprotein and cytochrome C which is not affected by steroids. The DPN-cytochrome C reductase of Mahler (which transfers directly from flavoprotein to cytochrome C) was prepared, studied under varied conditions, and found to be unresponsive to steroids. Direct assay of microsomal cytochrome reductase, cytochrome oxidase and DPNH and succinate dehydrogenase revealed no steroid inhibition. Under some (somewhat different) conditions, succinate oxidation could be inhibited.

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* Animals supplied by Dr. Laurence Corwin, NIAMD, NIH.

In an experiment designed to test for development of steroid tolerance, pretreatment of rats with large doses of steroids did not alter the activity of DPNH cytochrome C reductase or the responses to steroid or cytochrome C.

To explore further the relationship of this effect to tumor suppression we are studying several groups of steroid sensitive and steroid resistant mouse thymus tumors*. Preliminary results suggest that steroid resistance and sensitivity can be correlated with the DPNH oxidase activity of the tumor preparations. (THESE RESULTS ARE CONSIDERED PRELIMINARY AND CONFIDENTIAL).

Additional physiologic and pathologic correlates are being investigated.

Succinate Oxidation.

In fresh tissue homogenates and mitochondria succinate oxidation can be inhibited by steroids, but much higher (50 - 75 X) concentrations of steroid are required. Here the effect has not been completely localized, but does not appear to be between flavoprotein and cytochrome b. This effect is very labile in contrast with the stable DPNH-steroid relationship.

Lactate and Pyruvate Oxidation.

Studies were continued with steroid effects on both pyruvate and lactate oxidation.

A. Pyruvate oxidation: In liver homogenates and particular systems, the production of $C^{14}O_2$ from C_1 labeled pyruvate C^{14} is consistently inhibited by testosterone, DOC, androstene,3-17-dione, Δ^1 androstadien,3-17-dione, and to considerably lesser extent by a number of other steroids. This effect is observed under a variety of conditions, but is best seen with low concentration of tissue preparation, non-limiting concentrations of substrate, in the presence of from 10^{-3} to 10^{-2} M $MgCl_2$, and at pH 7.5-8.0. Fractionation studies revealed the effect to be most striking in the cell fraction sedimenting between 0-800 X G (comparable to activity of crude homogenate).

* Dr. Alexander Dmitrach is engaged in these investigations. Dr. Michael Potter of NCI is cooperating in the animal experimentation.

Addition of excess Coenzyme A, thiamine pyrophosphate, or DPN did not alter the effect. Inhibition was decreased by lipoic acid, oxalacetate, or arsenite.

Results with C_1 labeled lactate failed to reveal consistent inhibition.

Neither the site nor mechanism of inhibition have been elucidated.

D. Lactate oxidation: Using large concentrations of liver homogenate it was possible in about 70% of experiments to show 2-fold stimulation of $C^{14}O_2$ production from C_2 labeled lactate-C^{14} but not C_2 pyruvate by various steroids. Unfortunately, elaborate attempts to work out conditions under which the effect could be consistently observed were not completely successful.

Stimulation was diminished or abolished by malonate, pyruvate, oxalacetate or arsenite. It was generally unaffected by TPNH generating systems, or ADP. Under some conditions, the addition of DPN or DPNH enhanced the effect. It was particularly interesting that the addition of ultracentrifugate of the homogenate suppressed the control rate of CO_2 production and this suppression was relieved by steroids.

The physiologic implications of this somewhat fickle steroid effect have not been determined.

Adrenalectomized and gonadectomized rats did not appear to respond differently from the normals.

C_2 labeled propionate and C_1 and C_2 labeled acetate gave results similar to C_2 labeled lactate.

Serial No. NIAMD-12SC

PHS-NIH
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Part B.

Honors, Awards, and Publications

Publications other than abstracts from this project:

1) Yielding, K. L., and Tomkins, G. M.: An effect of enzymic reduction of steroids on triphosphopyridine nucleotide-dependent glucose-6-phosphate oxidation. Biochim.Biophys.Acta., in press.

2) Yielding, K. L., and Tomkins, G. M.: Inhibition of the enzymic oxidation of DPNH by steroid hormones. Proc.Nat.Acad.Sci., Dec., 1959, in press.

3) Yielding, K. L., and Tomkins, G. M.: Steroid sensitive and steroid insensitive electron transport pathways (Manuscript complete -- to be sent to J.Biol.Chem. as preliminary communication).

Serial No. NIAMD-1901
1. Clinical Investigations
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Juvenile Rheumatoid Arthritis

Principal Investigator: Dr. K. Lenone Yielding

Other Investigators: Drs. John Utz and Joseph J. Bunim

Cooperating Units: NIAID-58

Man Years (calendar year 1959):
Total: $\leq$ 1/3
Professional: $\leq$ 1/3
Other: 0

Project Description:

A. Search for infectious agent in juvenile rheumatoid arthritis.

B. Study clinical manifestations and course of rheumatoid arthritis in children.

Methods Employed:

As in project description. Blood, exudates, excretions, and tissues, when possible, were cultured exhaustively for viruses and bacterial pathogens.

Major Findings:

Of 16 patients studied clinically, only 5 satisfied the strict criteria of acute febrile activity and were cultured. Cultures have all been negative.

Part B included Yes [ ] No [X]

Serial No. NIAMD-1396
1. Clinical Investigations
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Enzyme Studies in Myotonia Congenita

Principal Investigator: Dr. E. Lemoine Yielding

(Tissue Taken from Patient at USPHS Hospital in Baltimore, Maryland by Investigator)

Other Investigators: Dr. Gordon M. Tomkins

Cooperating Units: There were no cooperating units elsewhere in the Public Health Service

Man Years (calendar year 1959):
Total: 1/3
Professional: 1/3
Other: 0

Project Description:

Objective:

To determine enzyme basis for myotonia.

Methods Employed:

Direct enzyme and chemical assays on muscle extracts.

Major Findings:

Using muscle extracts from one normal and one myotonic individual, ATPase and phosphopyruvichinase activities were determined as indices of ATP breakdown and formation, and expressed as activity/mg. of muscle protein. ATPase activities were identical but phosphopyruvichinase activity was almost doubled. (In the face of muscle hypertrophy it is not known which enzyme is basically changed).

Part B included Yes [ ] No [X]

Proposed Course of Project:

In order to pursue these interesting findings, a family of goats was obtained in which a myopathy essentially identical to human myotonia congenita occurs with high frequency. When clinical myotonia develops in these goats, it is expected to pursue the above findings.

(THIS DATA IS PRELIMINARY AND CONSIDERED CONFIDENTIAL).

Serial No. [illegible]
Clinical Investigations
Arthritis & Rheumatism Br.,
Bethesda, Maryland

PHS - NIH
Individual Project Report
Calendar Year 1959

Part A

PROJECT TITLE: Biochemical Aspects of Gastrointestinal Diseases

PRINCIPAL INVESTIGATOR: Leonard Lester

OTHER INVESTIGATORS: John W. Singleton

COOPERATING UNITS: None

MAN YEARS (Calendar Year 1959):
Total: 1-1/2
Professional: 1
Other: 1/2

PROJECT DESCRIPTION:

I. General Comments

The metabolic processes that transpire in the cells of the mucosal lining of the stomach, intestines and gall bladder are far from completely delineated, and consequently their interrelations with the physiologic activities of these areas are not fully understood. Although the biochemical reactions that occur within the liver and pancreas have been studied in greater detail, there remain many gaps in our knowledge of these organs, too. It is the long-term purpose of this unit to investigate pathways of metabolism of these tissues, using at first material from animal sources and eventually biopsy specimens from human subjects with and without diseases of these tissues. It is then intended to apply the information gained in these studies to the further investigation of the functions of these organs, in animals and humans, and to investigation of hormonal regulation of their functions.

The initial phase of this project entails the setting up of many methods for studies of tissue metabolism, and of animal and human physiology.

II. Studies of Enzyme Systems

A. Bile pigment interconversions

The enzymes that regulate the reaction sequence involved in the conversion of heme to bile pigments, and then to fecal excretory pigments, have not been studied extensively. Understanding of these enzymes may well bear on such subjects as the mechanisms underlying the production of jaundice and the enterohepatic circulation of bile pigment metabolites. We have initiated our study with the reaction in which biliverdin is reduced to bilirubin. Lemberg and Wyndham studied this reaction in 1936 and demonstrated reducing activity in many tissues of the guinea pig and in the livers of the many animals they studied. Their assay of activity was only qualitative, however. We have developed a quantitative assay for this activity and have shown the reaction to be dependent on DPNH. TPNH is soon to be tested. Our tentative formulation of the reaction is:

$$\text{Biliverdin} + \text{DPNH} + \text{H}^{+} \rightarrow \text{Bilirubin} + \text{DPN}^{+}$$

Additional evidence suggests that the reaction is enzyme catalyzed and we are now engaged in an attempt to purify the enzyme in order to study its properties. If purified enzyme can be prepared, a host of related investigations can be undertaken. These include the development of a specific enzymatic assay for biliverdin in blood, and the use of this reaction to generate bilirubin in vitro in order to study its metabolism in greater detail than is feasible at present.

B. d-Xylose metabolism

A clinical test for intestinal absorptive capacity, now in general use, involves the feeding of d-xylose and the determination of the quantity of xylose excreted in the urine during the ensuing five hours. It is assumed that xylose metabolism is not a significant variable in this "xylose tolerance test." Although the pathway of metabolism of d-xylose in mammals is not elucidated at present, Segal et al., have suggested that in man the liver metabolizes as much as 40% of intravenously administered d-xylose. In order to explore this further, we are studying the metabolism of xylose-1-C^{14} by tissue extracts and in the liver of rats during perfusion studies. Initial results suggest that in the rat the liver is not a site of major metabolic activity.

C. Source of human intestinal mucosa for enzyme studies

We are exploring the possibility of using intraluminal biopsy tubes to obtain samples of intestinal mucosa on which studies of enzyme activity and pathways of metabolism can be performed. We are currently engaged in perfecting our skill in the use of the Rubin biopsy instrument.

III. In vitro Physiological Studies

A. Transport functions

1. Isolated segment of small intestine - We have modified an apparatus used by others for the study of transport across isolated segments of guinea pig small intestine. We are still engaged in perfecting the technique. It is our intention to use it thereafter to study the effects of enzyme inhibitors, hormones, and other factors on the transport of various compounds across the small bowel mucosa.

2. Bacterial transport of fatty acid - Working with Dr. Nirenberg, we have isolated a soil bacterium that can utilize octanoic acid as a sole source of nutriment. We plan next to radiate this organism in an attempt to produce and isolate a mutant that no longer metabolizes octanoic acid, even though it retains the capacity to transport this fatty acid from the external medium into the cell. Such a mutant would permit us to investigate fatty acid transport in bacteria in some detail. It is hoped that understanding some aspects of the transport mechanism in bacteria will provide insight into similar functions in mammalian tissues.

B. Rat liver perfusion studies

In collaboration with Dr. Mortimore, who has developed a technique for perfusion studies of the isolated rat liver, we hope to study not only d-xylose metabolism (II-B) but also bile pigment metabolism, endocrine influences on various liver functions, and details of the enterohepatic circulation of various compounds. Preliminary studies are now in progress.

IV. Clinical Studies

A. Diagnostic and investigative techniques

The following determinations can now be performed in our laboratory:

(1). Fecal fat excretion; (2). Serum vitamin A and carotene levels; (3). Blood and urinary xylose levels for the xylose tolerance test; (4). Serum bilirubin levels, and (5) Urinary 5-hydroxy-3-indoleacetic acid (now being set up).

We have performed two intestinal biopsies, both successfully. We plan to set up methods for the determination of fecal excretion of I^{131}-triolein and I^{131}-oleic acid, as well as I^{131}-polyvinylpyrollidine.

B. Patients studied

1. Carcinoid syndrome due to metastatic carcinoid - We are studying patients with this syndrome to determine the frequency of intestinal malabsorption in the presence of elevated levels of blood serotonin. Two patients with functioning carcinoids and one with a metastatic tumor that may be a nonfunctioning carcinoid are now under study on the ward.

2. Hereditary disease of the gastrointestinal tract - This is being studied in an attempt to detect hitherto unsuspected biochemical lesions related to the digestive system. We have studied one member of a family with polycystic disease of the liver and/or kidneys.

Part B included: NO

Serial No. NIAMD-1330
Clinical Investigations
Arthritis & Rheumatism Br.
Bethesda, Maryland

PHS - NIH
Individual Project Report
Calendar Year 1959

Part A

PROJECT TITLE: Studies on Alcaptonuria and Ochronotic Arthritis in Man and Animals and on Phenylketonuria

PRINCIPAL INVESTIGATOR: Bert N. La Du

OTHER INVESTIGATORS: J. E. Seegmiller, V. Zannoni and M. O'Brien

COOPERATING UNITS: Dr. Richard Auld, Pediatrics Dept., Georgetown University Hospital, Wash., D. C.

MAN YEARS (Calendar Year 1959):
Total: 3-1/3
Professional: 2-1/3
Other: 1

PROJECT DESCRIPTION:

A. Alcaptonuria and Ochronotic Arthritis

Objectives in studying patients with alcaptonuria have been several: (1) to determine the exact nature of the metabolic defect in this condition; (2) to study the hereditary pattern of this disease, and, if possible, to develop a test which will detect the heterozygous state in relatives of alcaptonurics carrying the trait; (3) to study the formation and deposition of the pigment derived from homogentisic acid and to determine how it produces the pathological changes in the connective tissues, particularly the joints; (4) to study the cause of the arthritis nearly always associated with this condition, and (5) to attempt various means of treatment of this metabolic disease.

Clinical Studies:

Nature of defect in alcaptonuria - Quantitative analysis of the enzymes involved in tyrosine metabolism have been made in liver and kidney homogenates from autopsy specimens of another patient with alcaptonuria. Again it has been possible to show that alcaptonuric

tissues differ from the normal only in having no detectable homogentisic acid oxidase activity. Thus, it has been clearly demonstrated in two families that the defect in this metabolic disease consists of a deficiency of homogentisic acid oxidase activity in the liver and kidney.

Tissues of alcaptonuric patients - The concentration of homogentisic acid has been measured in knee joint synovial fluid of an alcaptonuric patient and of the rib cartilage of another patient with this disease. The levels in each of these tissues were lower than in the blood, indicating that homogentisic acid is not maintained at a high concentration within these tissues in alcaptonuria.

Inheritance of alcaptonuria - The recent suggestion of Milch and Milch that this disease is inherited as a dominant trait with incomplete penetrance, rather than as a simple autosomal recessive inheritance, is not supported by an examination of the pedigree of one of our patients. Upon careful questioning it was disclosed that there had been a consanguineous marriage which was not mentioned in earlier interviews. With this complete information, a simple recessive inheritance adequately explains the expression of the disease in this family.

Animal Studies:

1. Experimental ochronosis and arthritis in guinea pigs - As part of the study on the mechanism by which the accumulation of homogentisic acid leads to the development of ochronotic arthritis in alcaptonuria, the distribution of homogentisic acid in the tissues of guinea pigs has been measured at different times by a specific enzymatic method after the intraperitoneal injection of this acid. Very low concentrations were found in the muscle, liver and the other organs, but values almost as high as in plasma were present in the cartilage and skin. This unusual predilection of homogentisic acid for the connective tissues is in agreement with the deposition of the ochronotic pigment in these same areas. Further studies are being made of the nature and syntheses of the pigment and its relationship to the associated arthritis.

2. Enzymatic synthesis of homogentisic acid (Role of vitamin C in tyrosine metabolism) - Detailed studies of the enzyme system of liver which catalyzes the formation of homogentisic acid from p-hydroxyphenylpyruvic acid (the keto acid of tyrosine) are being continued. Vitamin C is involved in this enzymatic reaction and scorbutic guinea pigs have a defect in tyrosine metabolism and excrete p-hydroxyphenylpyruvic acid when fed this amino acid. The metabolic defect is corrected by vitamin C, but until recently

it was not known how this vitamin maintains normal tyrosine metabolism. Some insight into the mechanism came in studies with purified liver enzymes. It was found that ascorbic acid and 2,6-dichlorophenolindophenol had the property of protecting one of them, p-hydroxyphenylpyruvic acid oxidase (the enzyme which catalyzes the oxidation of p-hydroxyphenylpyruvic acid to homogentisic acid) from being inhibited by its substrate. In the presence of ascorbic acid, oxidation continued; in its absence, the oxidation slowed down and stopped. Recently we have been able to demonstrate the way the vitamin acts in vivo. Scorbutic guinea pigs were found to have as much p-hydroxyphenylpyruvic acid oxidase as normal animals, but when the scorbutic group was injected with p-hydroxyphenylpyruvic acid, over half of their liver p-hydroxyphenylpyruvic acid oxidase was inactive one hour later. In contrast, injection of the substrate did not inhibit the oxidase in normal guinea pigs. It appears that ascorbic acid acts in vivo to protect the oxidase from inhibition as was found in the enzyme studies in vitro.

It is of interest that scorbutic guinea pigs given 2,6-dichlorophenolindophenol several hours before an injection of p-hydroxyphenylpyruvic acid were also protected; thus, the dye has some anti-scorbutic activity. Further studies on the ability of the dye to correct other aspects of scurvy are in progress and histological examination of the tissues of dye treated animals is being made.

Chemical Studies:

1. Method for the estimation of homogentisic acid in synovial fluid and other tissues - The specific enzymatic method to measure small amounts of homogentisic acid in plasma has been modified to make it suitable for the analysis of homogentisic acid in tissues. The method has been utilized in studies of the distribution of homogentisic acid in the tissues of patients with alcaptonuria and in studies of the distribution of this acid in guinea pigs.

2. Measurement of homogentisic acid lactone and esters of homogentisic acid - The method for homogentisic acid has also been modified to measure the derivation of homogentisic acid, i.e., the lactone and methyl and ethyl esters. The metabolic fate and distribution of these derivatives has been studied in guinea pigs in the hopes that one of these compounds would be metabolized slowly to homogentisic acid and thereby maintain a higher plasma level over a longer period of time than when homogentisic acid itself is given. Such a compound is needed to induce experimental ochronosis in animals.

B. Phenylketonuria

Clinical Studies:

1. Best method of detection of heterozygous trait - Several analytical methods have been proposed to detect the carrier of the phenylketonuria trait; these include the fasting blood level of phenylalanine, the plasma level of phenylalanine after an oral phenylalanine tolerance test, or the ratio of the phenylalanine to tyrosine after an oral load of phenylalanine. In collaboration with Dr. Richard Auld of the Georgetown University Retarded Children's Clinic, we are comparing all these methods using our new analytical method to see which is the most reliable. The experimental group is composed of parents of phenylketonuric children being followed by the Clinic.

2. Effectiveness of diet low in phenylalanine in preventing mental retardation in phenylketonuric children - Studies on the effectiveness of the low phenylalanine diet in treating phenylketonuric children require repeated measurement of the level of blood phenylalanine and tyrosine. The variable response to the diet, particularly in older infants, may be largely due to the greater difficulty in maintaining a low level of blood phenylalanine in this group. We have been analyzing the blood each month of several children being followed at the Retarded Children's Clinic, Georgetown University Hospital, to evaluate the effectiveness of the diet.

3. Early diagnosis of phenylketonuria in newborn infant - A sibling born in a family with known phenylketonuria has a 1 in 4 chance of being affected. The new enzymatic method for blood phenylalanine developed in this laboratory, particularly the micro modification, makes it reasonable to make the diagnosis within a day or two after birth, and such analysis should be done in newborn infants in families with known phenylketonuria. We are presently doing serial analyses on blood samples on an infant with this background in order to make the diagnosis and start the special diet as soon as possible if this child should have phenylketonuria.

Chemical Studies:

1. Method for measuring aromatic amino acid analogues - The principle of the method to measure phenylalanine in blood has been adapted to measure small quantities of several other aromatic amino acids, such as p-fluorophenylalanine and β-thienylalanine, and the metabolism of the latter is being studied in guinea pigs. The effect of β-thienylalanine, an antimetabolite of phenylalanine, on the concentration of phenylalanine and tyrosine metabolites in blood and tissues is also being investigated.

Part B included: YES

Serial No. [illegible]

PHS - NIH
Individual Project Report
Calender Year 1959

Part B: Honors, Awards and Publications

PUBLICATIONS:

1. Zannoni, V. G., and La Du, B. N., The tyrosine oxidation system of liver. IV. Studies on the inhibition of p-hydroxyphenylpyruvic acid oxidase by excess substrate. J. Biol. Chem., 234: 2925-2931, 1959.

2. La Du, B. N., and Michael, P. J., An enzymatic spectrophotometric method for the determination of phenylalanine in blood. J. of Lab. and Clin. Med., In press. (Feb. 1960).

3. La Du, B. N., The importance of early diagnosis and treatment of phenylketonuria. Annals of Int. Med.

4. Zannoni, V. G., and La Du, B. N., Studies on the defect in tyrosine metabolism in scorbutic guinea pigs. J. Biol. Chem. In press (Jan. 1960).

5. La Du, B. N., Tyrosinosis, chapter in Biochemistry of Molecular Diseases, edited by J. Stanbury, J. B. Wyngaarden and D. Fredrickson. New York, McGraw-Hill. In press.

Serial No. NIAMD-133C
Clinical Investigations
Arthritis & Rheumatism Br.
Bethesda, Maryland

PHS - NIH
Individual Project Report
Calendar Year 1959

Part A

PROJECT TITLE: Metabolic and Therapeutic Studies of Gouty Arthritis and Hyperuricemia

PRINCIPAL INVESTIGATOR: J. E. Seegmiller

OTHER INVESTIGATORS: Arthur I. Grayzel, John J. Burns (NHI) and Peter Dayton (NHI)

COOPERATING UNITS: Laboratory of Chemical Pharmacology, NHI

MAN YEARS: (Calendar Year 1959)
Total: 1-1/2
Professional: 1
Other: 1/2

PROJECT DESCRIPTION:

Studies on drugs for the management of problem cases of gout have been continued. Previous work in the National Heart Institute on a group of compounds chemically related to phenylbutazone had shown that antirheumatic activity could be correlated with chemical structure and that uricosuric activity could be related to the acid association constant (pKa) of the drug. A urinary metabolite of phenylbutazone, oxyphenbutazone, had been shown to possess potent antirheumatic activity, but very little uricosuric activity. Upon the introduction of a keto group into the side chain of oxyphenbutazone, the resulting compound (G-29701) was found to possess potent uricosuric activity, but little antirheumatic activity. Again the uricosuric activity was correlated with an increased acidity of the compound with the pKa dropping from 4.6 for oxyphenbutazone to 2.3 for keto-oxyphenbutazone. There was a corresponding decline in the biological half-life from 72 hours to 8 hours which prevented the high serum levels needed for an antirheumatic effect with the parent compound. This drug has been given to 9 patients for short periods of time and has been very well tolerated, with no toxic side effects to date. Since the other potent uricosuric agents now available have a considerably shorter biological half-life, on the order of 3 hours, the 8 hour half-life

of keto-oxyphenbutazone gives promise of providing a more sustained uricosuric action with less frequent administration of the drug.

The antagonistic action of salicylates on the uricosuric effect of sexesolamine and sulfinpyrazone has been further studied and found to exist at even low doses of salicylates. By contrast, acetaminophen, which is the active metabolic product of acetanilid and acetophenetidin, gives no such antagonistic action, while at the same time providing an analgesic action.

A new method has been devised for determination of ammonia in biological fluids at physiological pH, utilizing glutamic dehydrogenase. Evidence has been obtained for the presence of free ammonia in normal human plasma.

Part B included: YES

Serial No. NIAMD-1497

PHS - NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards and Publications

PUBLICATIONS:

1. Liddle, L., Seegmiller, J. E., and Laster, L., The enzymatic spectrophotometric method for determination of uric acid. J. of Lab. and Clin. Med., 54: 903-913, 1959.

2. Crain, D., Epstein, W., Howell, D., Margolis, H., Phelps, E., Rawls, W., Rosenberg, E., Seegmiller, J. E., Shulman, W., Sokoloff, L., Thompson, T., and Toone, E., Primer on the Rheumatic Diseases, J. A. M. A., 171: No. 9, 1205-1220, 1959; 171: No. 10, 1345-1356, 1959; 171: No. 12, 1680-1691, 1959.

Serial No. [illegible]
Clinical Investigations
Arthritis & Rheumatism Br.
Bethesda, Maryland

PHS - NIH
Individual Project Report
Calendar Year 1959

Part A

PROJECT TITLE: Abnormalities of Purine Metabolism Associated with Gout

PRINCIPAL INVESTIGATOR: J. E. Seegmiller

OTHER INVESTIGATORS: Arthur I. Grayzel and Lois Liddle

COOPERATING UNITS: None

MAN YEARS (Calendar Year 1959):
Total: 2-1/2
Professional: 1-1/2
Other: 1

PROJECT DESCRIPTION:

The metabolic origin and disposition of uric acid in gouty patients has been studied further by administering isotopically labeled precursors of uric acid along with labeled uric acid itself and following the incorporation of label into urinary uric acid in normal and gouty subjects. A substantial portion of the patients with gout show an excessive synthesis de novo of uric acid as measured by the extent of incorporation of glycine-1-C^{14} into urinary uric acid. Additional patients can be shown to be producing excessive amounts of uric acid if the glycine incorporation data is corrected for the dynamics of the urate pool. This increased production does not show up in the urinary uric acid excretion values because of its extra-renal disposition. There still remains a portion of the gouty patients who show no demonstrable difference in the extent of glycine-1-C^{14} incorporation into urinary uric acid from that of normal individuals.

A pharmacological agent which suppresses the excessive uric acid synthesis found in some gouty subjects has been studied further. 6-Diazo-5-oxo-L-norleucine (DON) which has been shown in this laboratory to suppress uric acid synthesis in two gouty subjects, has been administered to a total of seven gouty patients. Two patients

who showed no drop in serum uric acid or in urinary uric acid excretion nevertheless showed a substantial reduction in the incorporation of glycine-1-C^{14} into urinary uric acid. This suppression of purine biosynthesis was evidently masked by the large urate pool in these subjects. Undesirable effects of DON consisted of duodenal ulcers in two patients and ulcerations of the oral mucosa in five of the seven patients studied. Routine use of this drug for suppressing the uric acid production in gouty patients appears to be imprudent. It is conceivable, however, that more specific inhibitors of purine biosynthesis might carry with them a more favorable therapeutic index.

An experimental tool for studying the homeostatic control of purine synthesis in the human has been found in the action of a drug, 2-ethylamino-1,3,4-thiadiazole, a nicotinamide antagonist which has been used experimentally in the treatment of cancer. Other workers noted that its use in the human resulted in an increase in both serum urate values and in daily urinary uric acid. The origin of this increased uric acid, whether from cellular breakdown or de novo synthesis, was not clear. We have been able to confirm this finding and furthermore to show that the increased uric acid production is the consequence of an increased purine biosynthesis induced by 2-ethylaminothiadiazole. The extent of incorporation of glycine-1-C^{14} into urinary uric acid in a non-gouty individual was brought up to the range observed in gouty subjects by administration of 2-ethylaminothiadiazole. Furthermore, its effect was completely prevented by administration of large doses of nicotinamide. This drug was found to have a comparable effect on urinary allantoin and uric acid production in the guinea pig and in vitro studies of its action are now underway.

Part B included: YES

Serial No. NIAMD-134C

PHS - NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

PUBLICATIONS:

1. Seegmiller, J. E., Grayzel, A. I., and Liddle, L., Excessive uric acid production in the human induced by 2-ethylamino-1,3,4-thiadiazole. Nature, 183: 1463-1464, 1959.

2. Wyngaarden, J. B., Seegmiller, J. E., Laster, L., and Blair, A., Utilization of hypoxanthine, adenine and 4-amino-5-imidazole-carboxamide for uric acid synthesis in man. Metabolism, 8: No. 4, 455-464, 1959.

3. Grayzel, A. I., Seegmiller, J. E., and Love, E., Suppression of uric acid synthesis in the gouty human by the use of 6-diazo-5-oxo-L-norleucine (DON). J. of Clin. Invest., In press (Mar., 1960).

Serial No. AM-1350
1. Clinical Investigations
2. Metabolic Diseases Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Studies in Bone Metabolism

Principal Investigator: Dr. G. Donald Whedon

Other Investigators: Dr. Leo Lutwak and Dr. Armen H. Tashjian, Jr.

Cooperating Units: None

Man Years (calendar year 1959):
Total: 6
Professional: 1
Other: 5

Project Description:

Objectives: 1. To investigate the factors affecting mineral storage and loss in demineralizing bone diseases, with particular attention to the relative influences of adrenal cortical steroids, gonadal steroids and dietary levels of minerals.

2. To investigate the rates of mineral deposition and amounts of bone undergoing active exchange with body fluids, in various bone disorders.

Methods Employed: 1. Metabolic balance studies under rigid dietary control in patients with various demineralizing bone diseases, noting the effects on nitrogen, calcium and phosphorus balances of adrenal cortical steroids, of gonadal steroids and of various dietary levels of calcium and phosphorus.

2. Determination of pool size, turnover rate and deposition rate of calcium in patients with various bone disorders, using tracer doses of radioactive calcium.

Part B included Yes

Major Findings: The major current research interest of this project is in determining the role of the level of dietary calcium intake in the pathogenesis and treatment of post-menopausal and senile osteoporosis. Data from dietary surveys by others, from our own metabolic balance studies extending over several months in each of several patients and from our own radioisotopic determinations of "bone formation rate" has led to the formulation of an expanded concept of the alteration in bone metabolism in osteoporosis, a concept which accords nutritional factors (particularly availability of mineral supplies to the skeleton) at least equal importance with previously recognized hormonal factors. This expanded concept (described in the Summary section of the Annual Reports) and the data currently available to support it are being published in this month's Federation Proceedings (transcript of Symposium on Effects of High Calcium Intakes, American Institute of Nutrition, held in April, 1959).

One facet of the results of these studies thus far hints at a probable significant part of the mineralization defect in certain osteoporosis patients. The generally higher calcium intake requirement in these patients to achieve calcium storage (much higher than for young normal individuals) and the considerable variability in this requirement suggest that an intestinal absorptive defect may be present in certain patients. Studies of fat and mineral absorption are being collaboratively initiated in these patients to assess this possibility.

Radioisotopic studies for the measurement of bone formation rate are continuing in patients with a variety of bone diseases to determine the influence of various hormonal and nutritional factors on bone metabolism. Currently special interest is being devoted to the effects of vitamin D in osteoporosis and to the calcium intake level in Paget's disease.

Significance to NIAMD Research: Senile and post-menopausal osteoporosis are twin forms of skeletal demineralization which are assuming increasing importance as the proportion of older people grows in the population of the U.S. and of the world. Surveys for incidence now in course seem to indicate that approximately 30% of women over the age of 50 years have roentgenographically visible osteoporosis of the spine. Careful review of results to date from management of these patients by hormonal therapy only, together with assessment of the investigations in this project to date, make it seem evident that long-accepted concepts are inadequate and a new approach is needed. Evaluation by these studies of the significance of nutritional factors in the pathogenesis and management of osteoporosis is indicating their importance with progressive weight.

Radioisotopic studies of bone formation rate are yielding data bearing on the fundamental differences in bone metabolism in various bone diseases and on the mode of action of numerous hormones and other agents on metabolic processes in bone.

Proposed Course of Project: Metabolic balance, isotopic and gastrointestinal absorptive (the latter collaboratively) studies will be continued in an effort to determine the effects and mode of action in calcium metabolism of adrenal cortical and gonadal steroids and of the mineral level of the diet, and also to obtain understanding of the processes of bone formation and resorption in various bone disorders.

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Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1) Lutwak, L.: The estimation of radioactive calcium-45 by liquid scintillation counting. Anal. Chem. 31:340, March 1959.

2) Whedon, G. D.: Present concepts of the physiology of bone in the aging human: influence of hormonal and other factors in osteoporosis. Proceedings of the Fourth International Congress of Gerontology. II:615-627, Tipografia Tito Mattioli, Fidenza, Italy, Feb. 1959.

3) Whedon, G. D.: Effects of high calcium intakes on bones, blood and soft tissues. American Institute of Nutrition Symposium on The Effects of High Calcium Intakes. Fed. Proc., Dec. 1959.

4) Whedon, G. D.: Osteoporosis: atrophy of disuse. Special book publication of research conference "Bone as a Tissue" held at the Lankenau Hospital, Philadelphia, Pennsylvania, October 30-31, 1958. (in press).

Serial No. NIAMD-136
1. Clinical Investigations
2. Metabolic Diseases Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Total Energy Metabolism: Studies in Health and Disease

Principal Investigator: Dr. G. Donald Whedon

Other Investigators: Drs. E. R. Buskirk, R. H. Thompson, R. Moore, L. Lutwak and A. Tashjian, Jr.

Cooperating Units: NIAMD, Laboratory of Nutrition and Endocrinology (Section on Nutrition); NCI, General Medicine Branch (Metabolism Service); NIMH, Laboratory of Clinical Science.

Man Years (calendar year 1959)
Total: 5-2/3
Professional: 3-2/3
Other: 2

Project Description:

Objectives: 1. To establish a technique of total energy balance which can be applied to various clinical problems and to fundamental physiological problems of energy metabolism not now understood.

2. To study the influence on total energy consumption and balance of various factors, including climate and the endocrine hormones.

3. To investigate the characteristics of energy balance and their influence on the nutritional state of patients in pertinent disease conditions, such as obesity and cancer.

Methods Employed: Indirect human calorimetry by means of complete continuous expired air analysis in the Metabolic Chamber, metabolic balance determinations, caloric analysis of dietary intake and excreta.

Part B included Yes

Major Findings: 1. Change in concept of a basic metabolic phenomenon: Last year's Metabolic Chamber study of the influence of cold environment on the metabolic effect of food (specific dynamic effect or SDE) produced such an unexpected finding that the experiments were repeated in additional young male subjects, using instrumentation with improved sensitivity. The added studies have confirmed the finding first reported by these NIAMD investigators that human beings differ greatly from dogs in the utilization of thermogenesis associated with eating for body heat balance. The studies on dogs, performed by Rubner during the classic period of calorimetry investigation, had shown that food-induced thermogenesis would replace cold-induced thermogenesis and animals fed in the cold would be kept from shivering. The current human studies, on the other hand, have shown summation of the two types of thermogenesis; that from food did not replace that from cold. It will be necessary to modify the statements in most Physiology textbooks on cold-SDE thermogenesis interrelationships, which suggest that Rubner's work is applicable to all homeothermic species, and indicate inter-species differences.

2. Delineation with fidelity of moment-to-moment metabolic changes reveal characteristic features of phenomena obscured by older methods: The unique capacity of the Metabolic Chamber's instrumentation for tracing the patterns of fundamental physiological phenomena has been demonstrated in this study of the influence of cold on SDE. Not merely the degree of energy expenditure but the kaleidoscopic form can be outlined by the Chamber's system of continuous expired gas sampling for minimal changes in oxygen and carbon dioxide concentration, in conjunction with continuous recording of other physiological data such as body temperature at various sites, heart rate, etc. Various methods applied in the past to human energy studies have all been based on interval sampling of expired air which totally obscures moment to moment metabolic changes. Although cyclic variation in oxygen consumption associated with shivering has been suggested in previous interval sampling studies, delineation of metabolic changes with fidelity which is provided by the Chamber continuous sampling procedure has made possible the following observations in studies thus far: 1) determination of the exact duration of and interval between various bursts of energy expenditure associated with shivering, 2) recognition of a sustained underlying increase in metabolism in the cold distinct from the periodic peaks associated with gross body shivering, 3) the finding of marked inter-individual differences in the metabolic response to cold, both in lag before initiation and in magnitude attained, 4) definition of the duration and total amount of metabolic change associated with ingestion of food (SDE), and 5) accurate separation of the metabolic responses due to SDE and to cold, and recognition of an altered SDE metabolic pattern in the cold as compared with its form in a comfortable environment. Comparison of continuous body temperature measurements

with oxygen consumption emphasized the large capacity of the body for thermal damping. Cyclic changes in oxygen consumption were not reflected by changes in any of the measured body temperatures.

3. Collaborative respiratory-metabolic and biochemical studies of exercise and obesity: It is anticipated -- with support for this anticipation from preliminary observations -- that delineation of patterns of metabolic response to exercise, in association with biochemical measurements, will provide insight into alterations in metabolic processes in states of impaired physical, cardiovascular or metabolic function. Such combined respiratory-metabolic and biochemical studies in collaboration with three other metabolic research groups, have been initiated in normal subjects and in patients with moderate to marked obesity in an effort to appraise the metabolic shifts or alterations occurring in response to exercise and to caloric restriction. As an example of the new areas being investigated in what is now an early phase of these dynamic studies may be cited the elevations noted in serum ketone body and unesterified fatty acid levels during the weeks of walking exercise. These biochemical changes hint that during long periods of continuing moderate exercise there may be set in motion increases in the processes of fat mobilization and possibly fat metabolism. Studies reported last year by this group showed the pronounced effects on carbohydrate metabolism from prolonged inactivity, reversible by extended exercise.

4. Modification and refinement of method of determination of total body water. One of the measurements made during the course of the various energy balance studies is total body water, an important component of body composition. Although several methods are available for the determination of total body water, only the dilution procedure using tritium-labeled water appeared attractive from the points of view of validity, reproducibility and available instrumentation (liquid scintillation counter). In order to use the tritium method it was necessary to modify existing procedures to separate conveniently the diluted tracer from the body fluid sample. A method involving low temperature, low pressure sublimation has been adopted and has proven quite satisfactory. Shell-frozen samples of tritium-labeled plasma or urine are frozen at 26°C and a pressure of 1000 microns of mercury. The sublimate is condensed in a trap at -70°C, thawed, and radioassayed in the liquid scintillation counter. Care must be taken to complete the sublimation process because a serial sampling experiment indicated an enrichment of the tracer as sublimation progressed to completion. Errors of -10% body water could result if this precaution is not observed.

5. Instrumentation research: Initiated for the purpose of altering industrial continuous flow gas analyzers specifically for Metabolic Chamber research (50-fold increase in sensitivity desired), efforts to improve the stability and sensitivity of the oxygen analyzer can now be considered an instrumentation research accomplishment. This is manifested by the desire of two other research groups (not at NIH) to adopt the circuit modification now in use in Metabolic Chamber studies. Aided by advice from the NHI Laboratory of Technical Development, the oxygen instrument has been refined to the point where 0.02% changes in oxygen concentration can be accurately detected in air streams of 100 liters per minute. The carbon dioxide analyzer was modified some time ago and has performed satisfactorily over the past two years, but steps are under way to improve this cell even further. The staff of the Chamber has also developed a data handling system to deal with the voluminous data generated on the strip-chart recorders and to facilitate calculations; paper tape from the system will be fed to the NIH-IBM computer facility. This originally designed system has just been installed and is currently under test.

Significance to NIAMD Research: Studies of human energy metabolism in the Metabolic Chamber are concerned with a variety of basic problems of physiology and metabolism and are thus directly related to the principal interests of NIAMD research.

The study on specific dynamic effect and the influence thereon of cold environment represents investigation of a fundamental physiological phenomenon of energy metabolism. A distinct species difference was shown on the part of human subjects from dogs on which the classical studies of Rubner had been performed; the difference revealed in this study will require revision of the opinion of physiologists held since Rubner that thermogenesis from food ingestion can readily substitute for that from cold (shivering) in maintenance of body heat balance. Respiratory-metabolic studies of metabolic response to exercise in normal and obese subjects, with associated biochemical measurements, will provide insight into metabolic processes in various states of impaired physical, cardio-vascular or metabolic function. Instrumentation research is providing modifications of respiratory gas analytical apparatus which will be useful to other investigators in this and related fields.

Proposed Course of Project: Studies will be continued along the principal lines described in this report.

Serial No. MRAMB-1959

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1) Whedon, G. D. New human energy metabolism research. J. Am. Dietetic A., 35:683, No. 7, July, 1959.

2) Grande, F., Monagle, J.E., Buskirk, E. R. and Taylor, H. L.: Body temperature responses to exercise in man on restricted food and water intake. J. Appl. Physiol. 14:194, 1959.

3) Kreider, M.B., Iampietro, P. F., Buskirk, E. R. and Bass, D.E.: Effect of continuous cold exposure on nocturnal body temperatures of man. J. Appl. Physiol. 14:43, 1959.

4) Iampietro, P.F., Goldman, R. F., Buskirk, E.R., and Bass, D.E.: Response of Negro and white males to cold. J. Appl. Physiol. 14:798, 1959.

5) Bass, D.E., Iampietro, P.F., and Buskirk, E.R.: Comparison of basal plasma and blood volumes of Negro and white males. J. Appl. Physiol. 14:801, 1959.

6) Goldman, R. and Buskirk, E.R.: A method for underwater weighing and the determination of body density. Human Biology.(in press).

7) Buskirk, E. R. and Counsilman, J.E. Special exercise problems in middle age. Chapter in Science and Medicine of Exercise and Sport. Harper and Bros. (in press).

8) Moore, R. and Buskirk, E. R.: Exercise and body fluids. Chapter in Science and Medicine of Exercise and Sport. Harper and Bros. (in press).

9) Buskirk, E. R.: Underwater weighing. Human Biology.(in press).

10) Buskirk, E. R.: A discussion of problems related to the caloric cost of living. Bulletin of the New York Academy of Medicine.(in press)

Serial No. NAAMD-1570
1. Clinical Investigations
2. Metabolic Diseases Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Metabolic Effects of Adrenal Cortical Steroids

Principal Investigator: Dr. G. Donald Whedon

Other Investigators: Dr. Leo Lutwak and Dr. Armen H. Tashjian, Jr.

Cooperating Units : This project complements (and is cooperative with) "Trial of New Anti-Rheumatic Drugs."

Man Years (calendar year 1959):
Total: 1-1/3
Professional: 1/3
Other: 1

Project Description:

Objectives: To evaluate the metabolic effects of various new synthetic adrenal-cortical steroids with respect to sodium, potassium and nitrogen excretion and in selected instances with respect to calcium and phosphorus balance. Effective anti-inflammatory action does not qualify a new steroid for wide clinical trial in rheumatoid arthritis unless certain metabolic side-effects can be shown to be minor or absent. The particularly undesirable effects most often encountered are sodium and water retention, and potassium and nitrogen loss.

Methods Employed: Under rigid dietary control short-term metabolic studies (six weeks) are made of the effect of new synthetic adrenal steroids on the urinary excretion of nitrogen, sodium and potassium and on the blood levels of the latter two elements. When short-term studies suggest acceptability of the compound with respect to the metabolism of these elements, more lengthy studies are carried out in selected patients for the long-term effects of the steroids on the complete metabolic balance of these elements and of calcium and phosphorus.

Part B Included No

Major Findings: During the past year a single 90 day balance study on a patient with active rheumatoid arthritis has been conducted of the metabolic effects of a new synthetic steroid, compound 128, a 6-α-fluoro inated cortisone compound. This effective anti-rheumatic compound caused only a temporary diuresis of sodium and a modest increase in potassium excretion so that, if additional studies were to show similar results, the minimal degree of electrolytic side effects would tend to encourage further therapeutic trials. The striking finding with this steroid, however, was reduction in urinary calcium, a change not previously noted with any adrenal steroid yet subjected to metabolic balance assay. Completion of balance analyses are eagerly anticipated and further studies planned because of the great value which would result if a calcium-storing adrenal steroid should be conclusively identified.

Significance to NIAMD Research: This study is cooperative with "Trial of New Anti-Rheumatic Drugs," NIAMD, and is important primarily in indicating whether effective anti-rheumatic steroids may be safely given to patients over considerable periods of time with respect to metabolic effects. Of additional importance is the fact that determination of the metabolic action of steroids under study may yield information which will give useful leads to chemists engaged in the synthesis of various cortisone-like steroids.

Proposed Course of Project: This project will be continued intermittently along the present lines as facilities permit, with particular stress on electrolyte effects of the new steroids and attention to the long-term mineral effects, particularly of those compounds which appear destined for broad clinical use.

Serial No. HLMB-1390
1. Clinical Investigations
2. Metabolic Diseases Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Study of the Normal and Abnormal Physiology of the Formed Elements of the Blood

Principal Investigator: Dr. Frederick Stohlman, Jr.

Other Investigators: None

Cooperating Units: None

Man Years (Calendar year 1959):
Total: 1
Professional: 2/3
Other: 1/3

Project Description:

Objectives: Study of factors contributing to the production and destruction of formed elements of the blood in normal and disease states.

Methods Employed: Aside from routine determination of formed elements in the peripheral blood, these consist of measurement of red cell survival with Cr^{51} and differential agglutination, red cell production with Fe^{59} uptake.

In addition, the ability of the marrow of patients with refractory type anemias to respond normally to standard stimuli, phlebotomy, hypertransfusion and steroids is being studied.

Assays for erythropoietine from suitable patients before and after various forms of treatment are being conducted in conjunction with our basic research project.

Part B included Yes

Major Findings: 1. Correlation of plasma and urinary erythropoietine levels with bone marrow erythroid cellularity. It has been established that this relationship holds even in those instances in which there is a hypercellular marrow which fails to eventuate in the delivery of red cells.

2. In a study of patients with refractory anemia and abnormal erythropoiesis it has been established that (1) the erythroid elements are turning over at a normal to accelerated rate with death of cells in marrow, (2) the marrow responds normally to physiologic stimuli.

Significance to NIAMD Research: Anemia is a common complication of arthritis and certain metabolic diseases and may be refractory to treatment. A better understanding of the regulation of erythropoiesis is of basic interest and should eventually result in improved therapy.

Proposed Course of Project: Investigation of patients with polycythemia vera, anemia associated with rheumatoid arthritis or thalassemia with respect to the relationship of hypoxia and for a postulated red cell feedback in controlling red cell production.

Further study of refractory anemia and the effectiveness of steroids in therapy.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Stohlman, F., Jr.: Erythropoietine. Pediatrics 23: 935-936, 1959.

2. Stohlman, F., Jr.: Observations on the physiology of erythropoietine and its role in the regulation of red cell production. Annals of the New York Academy of Science. 77: 710-724, 1959.

Serial No. [illegible]
1. Clinical Investigation
2. Metabolic Diseases Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis.

Principal Investigator: Dr. N. R. Shulman

Other Investigators: Drs. T. C. Bithell, A. Leitner and R. Aster.

Cooperating Units: Dr. John Z. Hearon, Office of Mathematical Research; Drs. R. K. Shaw, J. D. Davidson, W. Rall, and Emil Frei, Cancer Chemotherapy Section, NCI.

Man Years (calendar year 1959):
Total : 3-1/4
Professional: 1-3/4
Other : 1-1/2

Project Description:

Objectives: Studies of the reactions and interactions of coagulation factors in vitro and in vivo to define further the nature of the blood coagulation mechanism and the factors of significance in the pathogenesis of obscure coagulation disorders in order to develop better methods of clinical therapy.

Methods Employed: Techniques of protein purification and characterization including plasma fractionation for specific coagulation factors and electrophoretic and ultracentrifugal analysis. Enzymology of proteolytic enzymes and their inhibitors including techniques required for refined kinetic analyses using protein and synthetic substances. All research techniques for quantitative measurement of the various coagulation factors. Various techniques of inorganic and organic chemistry. Pharmacologic and physiologic techniques applied in man and animals.

Part B included Yes

Major Findings: 1. Studies of the initial stages of blood coagulation. The combined work of many investigators indicates that at least five and possibly eight or more different coagulation factors interact during the initial stages of blood coagulation to produce thromboplastic activity, the activity which converts prothrombin to thrombin. Diseases caused primarily by abnormalities in thromboplastic activity are the different types of hemophilia and the so-called hemophiliaid states. It has been possible to identify the numerous factors involved in formation of thromboplastic activity; five different congenital hemorrhagic diseases with clotting defects related to an abnormality in thromboplastic activity have been attributable to a different specific deficiency in each case. In spite of the fact that many factors have been implicated in the formation of thromboplastic activity, there is remarkably little information concerning the biochemistry of thromboplastin formation or its activity. For instance, it is not known whether the various factors act in sequential enzymatic steps or combine stoichiometrically to form thromboplastin, whether thromboplastin acts only enzymatically on prothrombin or combines with it stoichiometrically as well, or whether the so-called "deficiencies" of thromboplastic substances represent a true lack or the presence of abnormal antagonists. Research directed at these problems has led to the following findings during the past year:

a. Following our finding (see 1958 report) that two of the factors involved in thromboplastin formation, anti-hemophilic globulin (AHG) and Factor V, could be irreversibly inactivated in vitro by agents which strongly bind calcium (e.g. ethylenediamine-tetra-acetic acid (EDTA)), we have shown that it is possible to make animals artifically deficient in these factors by exchange transfusion with blood treated with EDTA. This has permitted for the first time an evaluation of the turnover rates of these factors in "normal" animals. The only previous information on the half-life of AHG and Factor V has been obtained by measuring the survival of these materials in congenitally deficient patients; but the values obtained have been uncertain, for example, because it has been impossible to assess the effects of diffusion of these factors into extravascular spaces. By comparing results we obtained in animals having acutely-induced deficiency states with results we obtained in patients having congenital deficiency states, it was found that the half-life of these factors (approximately 8 hours) was similar to the rate at which they could be returned to the circulation in normal animals, indicating so far that the turnover rate of these substances can be extremely rapid even in normal animals. Studies of the survival of two other factors involved in thromboplastin formation, plasma thromboplastin component (PTC) and Factor VII, in

congenitally-deficient patients, showed that the half-life of these substances was also in the order of 8 hours. The implications are that all of the materials involved in thromboplastin formation are rapidly utilized *in vivo* regardless of their *in vitro* stability; for AHG and Factor V are extremely labile *in vitro* and consume rapidly when blood clots, whereas PTC and Factor VII are extremely stable *in vitro* and present in as high concentration in serum as plasma. It is interesting that all four of these thromboplastic factors have an *in vivo* turnover rate approximately 6 to 12 times faster than the turnover rate of clotting factors not involved in thromboplastin activity (e.g., prothrombin and fibrinogen). Further studies of this type promise to provide the type of information which is necessary in order to develop better methods of treating hemophilia and allied conditions, and to provide clues as to the biochemical nature of the initial stages of blood coagulation.

b. In addition to the theoretical implications this work has provided some very practical information concerning the use of the anti-coagulant, EDTA, in obtaining blood for transfusions into human beings. The finding that animals can be made artificially deficient in AHG and Factor V with EDTA blood indicates that EDTA should not be used to obtain blood for patients with AHG or Factor V deficiency or for patients who will receive massive transfusion (such as heart pump cases) because in the former instances the blood collected in EDTA would not correct the deficiency and in the latter instance the transfused blood could induce a serious hemorrhagic state.

c. Stemming from our observations (see 1958 report) that calcium is an integral part of the AHG and Factor V molecules, we have been measuring the calcium content of different plasma fractions in order to determine whether it is feasible to detect the specific clotting factors on the basis of their calcium content. The methodology of plasma fractionation and micro determination of calcium has been worked out and preliminary results indicate that it may be feasible to detect specific deficiencies of clotting factors by calcium determination alone and possibly follow changes in the distribution of plasma calcium in different protein fractions during blood coagulation. These studies may prove to be helpful in relating molecular structure to function of clotting factors and in determining the nature and sequence of biochemical reactions which take place during the formation of thromboplastin.

2. Clinical studies of unusual coagulation disorders.

a. Although the half-life of Factor VII in a congenitally deficient patient was found to be only 8 hours (see section 1.a. above) it has been found that benefits from administering plasma to such a patient last for 3 to 4 days after all traces of the administered factor have disappeared from the circulation by in vitro tests. Although we do not understand the meaning of this, we have been able to maintain prophylactic therapy by weekly infusions of plasma in a patient who otherwise invariably bleeds, and therefore have indications that concentrates of Factor VII may provide practical maintenance therapy for such patients. Because Factor VII and prothrombin are both reduced by dicumarol therapy, there have been suggestions that these two factors may be derivatives of the same precursor. It was interesting that massive doses of vitamin K_1 did not produce improvement in our Factor VII deficient patient in contrast to its therapeutic effectiveness in congenital prothrombin deficiency.

b. The mode of inheritance of Factor VII deficiency was studied by surveying the family of a Navajo Indian patient with this disease which necessitated a field trip to collect blood samples and obtain a careful family protocol on the Arizona Reservation. Our finding that this disease is non-sex linked and dominant with variable penetrance is in agreement with the one other genetic study of congenital Factor VII deficiency.

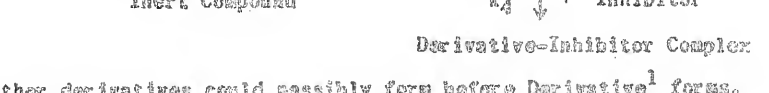
c. Study of several patients who developped unusual hemorrhagic manifestations while on dicumarol drugs, although adequately controlled as determined by prothrombin time values, showed that they had, in addition to the usual Factor VII and prothrombin deficiency, a deficiency of PTC and an abnormality in the thromboplastin generation test which suggested the lack of an additional factor as well. There are conflicting reports in the literature concerning the factors which may occasionally become deficient during dicumarol therapy. Our studies indicate that PTC as well as Factor X are affected and that these factors remain depressed long after prothrombin and Factor VII return to normal after discontinuing dicumarol.

d. We have been intensively investigating an unusual coagulation abnormality which has proven to be an acquired complete AHG deficiency without an anti-AHG antibody in an elderly female patient who has no detectable underlying disease. Since all other cases of AHG deficiency occur either congenitally in males or in females who have developed an antibody against the factor following pregnancy or in association with lupus erythematosus, the clinical and laboratory information obtained on this patient may lead to a further understanding of the role of AHG in blood coagulation and possibly provide information concerning the control of plasma levels of this factor.

e. In the course of evaluating new chemotherapeutic agents, the Cancer Chemotherapy group of the NCI found that the drug 4-amino-pyrazolo-pyrimidine (4APP) produced marked prolongation of the pro-thrombin time in treated patients. Our investigations of this abnormality showed that 4APP produced an acute transient drop in prothrombin, Factor V, and Factor VII concentrations which could not be prevented by massive doses of vitamin K_1 and which could be attributed to hepatocellular damage. Apart from establishing the precise nature of the toxic effect of this drug, these observations are of research interest because 4APP may prove to be an excellent agent for producing controlled specific deficiencies in laboratory animals.

f. The high incidence of thromboembolic complications in patients receiving steroid therapy have been attributed by some investigators to an elevated level of Factor VII. Using a new more sensitive technique which we devised for measuring Factor VII we found that Factor VII remained perfectly normal as did all other known coagulation factors as well as platelets in patients and control subjects given high doses of steroid hormone. Although we have not yet found the reason for the apparent hypercoaguable state, we know that previous explanations offered are untenable.

3. Kinetic studies of prothrombin conversion. When prothrombin is transformed into thrombin by biological activators there is little if any change in its physical properties, for so-called biothrombin has practically the same molecular weight and electrophoretic mobility as prothrombin. Therefore, it had not been possible to conclude from physico-chemical studies that transformation of prothrombin into biothrombin involves formation of other prothrombin derivatives. The kinetic studies done in association with Dr. J. Z. Hearon, which demonstrated the formation of several prothrombin derivatives during the conversion of prothrombin to thrombin (see 1958 report), have been extended and refined during the past year. The combined experimental and mathematical analysis of the prothrombin conversion system and its inhibition by proteolytic enzyme inhibitors has resulted in the following basic model for the reactions:



Other derivatives could possibly form before Derivative^1 forms. Rate constants k_1, k_2, and k_3 vary with the concentrations of con-version factors whereas k_4 is the unchanging rate constant of complex formation. The kinetic details of this unique form of competitive inhibition will be described in Dr. Hearon's report. The fact that such

derivatives form accounts for a number of the puzzling attributes of prothrombin conversion in biological systems which hitherto were explicable only by assuming that certain stoichiometric steps were involved rather than that they were purely enzymatic reactions. The implications are that the several prothrombin derivatives may have separate biochemical and physiologic functions.

Significance to NIAMD Research: 1. The types of *in vitro* and *in vivo* coagulation studies being done represent a fundamental approach to the understanding of the nature of diseases of hemorrhage and thrombosis. These diseases comprise a major segment of hematologic disorders, which have been a categorical interest of NIAMD. Further progress in diagnosis and treatment of a number of hemorrhagic diseases depends on understanding the nature of the metabolism and interaction of various coagulation factors. Such studies are appropriate to this Institute and have direct bearing on general problems concerning the metabolism of physiologically active protein.

2. Studies of the effects of proteolytic enzymes and their inhibitors on prothrombin to thrombin conversion have continued to demonstrate the great value of collaborative mathematical analysis. The conclusions reached have resulted from a combined experimental and mathematical analysis in which mathematics has not only proven the validity of the working hypothesis drawn from laboratory investigation, but has provided additional conclusions which have been confirmed experimentally. The mathematical analysis has also suggested new leads for further research.

3. In the studies of calcium-binding agents in relation to coagulation factors, the demonstration that at least two of the most labile coagulation factors contain calcium as an integral component suggests that further studies directed at relating molecular structure to function may prove valuable in unraveling some of the complexities of blood coagulation. The production of specific deficiencies of blood coagulation factors *in vivo* in laboratory animals using Na_2EDTA as an inactivating agent will facilitate experimental analysis of the pathogenesis and therapy of the naturally-occurring disorders. The turnover rates which have been established for the several clotting factors studied not only provide a useful practical guide in clinical therapy, but also have provided leads for further investigation of the biochemical processes involved in the initial stages of blood coagulation.

4. Consultation on coagulation problems throughout the Clinical Center has provided specialized clinical and laboratory service in an important area of medicine and has furnished a number of interesting cases for hematologic research study as well.

Proposed Course of Project: Studies of the biochemical and biophysical characteristics of the reactions involved in thromboplastic formation and the conversion of prothrombin to thrombin will be continued along the lines indicated in the present report.

Serial No. NIAMD-139C

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Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Shaw, R. K., Shulman, N. R., Davidson, J. D., Rall, W. and Frei, E. Studies with the experimental anti-tumor agent 4-amino-pyrazolo-pyrimidine, Cancer (in press).

Serial No. NIAMD-1406
1. Clinical Investigation
2. Metabolic Diseases Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A.

Project Title: Study of the Immunology of Blood Cell Deficiencies.

Principal Investigator: Dr.N. R. Shulman

Other Investigators: Drs. R. Aster, A. Leitner and T. Bithell

Cooperating Units : Dr. John Harris, Cleveland Metropolitan General Hospital.

Man Years (calendar year 1959):

Total	:	3-1/4
Professional	:	1-3/4
Other	:	1-1/2

Project Description:

Objectives: To study the pathogenesis and biochemistry of immunologic diseases which are caused by antibodies formed against autologous blood cells, and to determine the significance of this type of immunity in idiopathic blood cell deficiency states. Of special interest are the biochemical reactions which result in formation of complexes between cells, antibodies and drug haptenes, and the physiologic processes which result in sequestration of cells with attached antibodies.

Methods Employed: Techniques of quantitative immunochemistry including preparation and physico-chemical characterization of purified antibodies, micro-analyses for nitrogen, histamine, and alkaloid drugs, precise measurements of complement fixation, and quantitative measurements of cellular agglutination and lysis. Methods of provoking antibody responses in man and animals, methods of separating specific cell types from whole blood, electrophoresis, isotope tagging techniques, and Coon's fluorescent tagging techniques are used.

Part B included Yes

Major Findings: "Auto-immunity" (antibodies formed in an individual which react with the individual's own tissues) has been implicated with increasing frequency as the basis of diseases involving cellular destruction. Some of the most incisive examples are hematologic diseases in which a single type of circulating blood cell is destroyed by a specific antibody which appears to react with one particular cellular antigen. Although these hematologic immune diseases are relatively well defined, there are a number of major questions which have not yet been answered. For instance, do antibodies really develop against substances which have always been present in the individual; do some antibodies formed against truly foreign antigens attach to cells, not by forming a specific antigen-antibody complex, but by a more fortitious process of non-specific adsorption on a receptive cell surface; or indeed are certain somatic antigens essentially foreign to antibody-forming tissues? Our studies of hematologic auto-immune diseases have been directed at answering these and similar questions.

1. Comparison of antibody reactions in drug hemolytic anemia and drug thrombocytopenic purpura. Following our finding (see 1958 report) that the complex reactions which take place between quinidine, antibody, platelets, and complement in quinidine thrombocytopenic purpura are the same as the reactions which take place between stibophen, antibody, red cells, and complement in stibophen hemolytic anemia, we have continued work with the rare antibody induced by stibophen in an attempt to resolve the question of antigen specificity. Because we have had a very limited supply of serum containing antibody at 10^{-9} M concentration or less, these studies have required development of methods for measuring extremely low concentrations of catechol (stibophen and sodium catechol disulfonate) by spectrophotometric, spectrophotofluorometric, and isotopic labeling techniques combined with immuno-electrophoresis for application in determining the kinetics of antibody complex formation. Results so far indicate that the first step of the over-all reaction which results in an antibody-drug-cell-complement complex is the attachment of drug to antibody. This is an important finding, for if the first step of complex formation is combination of antibody with drug rather than cell with drug, the implications are that the cell-drug complex is not the antigen but that the antibody-drug complex may be non-specifically adsorbed on cell membranes just as other non-antibody plasma proteins are adsorbed.

Another very interesting finding pursuant to our observation that human but not guinea pig complement is fixed by stibophen-antibody-cell complexes was that only the second component of complement is fixed in the reaction. Complement components 1, 3 and 4 are not fixed and not involved in the hemolysis produced by this particular

antibody. The fixation of a single complement component (C'_2) by a hemolytic antibody is a unique reaction in immunology, and continued study of this reaction promises to provide further information concerning the chemisty and significance of the different complement components.

Other information obtained in this study was that in the order of 100 molecules of stibophen antibody per cell are necessary for cellular agglutination, that 1,000 or less antibody molecules attached with complement per cell are necessary for hemolysis; and that attachment of small amounts of complement (too little to cause hemolysis) prevents the cells from agglutinating even in the presence of large amounts of antibody. Further studies of this type will provide information concerning the physiological significance of agglutinating versus complement-fixing antibody complexes.

2. Pathogenesis of a newly recognized purpuric disease caused by sensitization of the patients' skin to their own red cells. In our 1958 report we presented details of studies of an unusual form of autosensitivity in which a minute amount of the patient's own red cells produced large painful ecchymoses when extravasated into the skin. We found that these lesions, which were produced by as little as 6 micrograms of red cell stroma, could be precisely duplicated by intradermal injections of as little as 1 microgram of histamine or by injection of any agent which released skin histamine (such as basic amines or trypsin). The conclusions were that ecchymoses were mediated by histamine released as the result of an antigen-antibody reaction occurring intradermally. Since then we have had opportunity to study another patient with a similar disorder in whom large painful ecchymoses were produced by intradermal injections of as little as 2 micrograms of red cell stroma. However, this patient did not develop ecchymoses when histamine or histamine-releasing agents were substituted for red cells. Various attempts to actually measure antigen-antibody combination in both cases by the most sensitive biological assay techniques available have been unsuccessful. Because fixed tissue antibodies do not lend themselves readily to *in vitro* analysis, further studies of spectrum of manifestations which are present or can be provoked in patients with autoerythrocyte sensitization may shed some light on the nature of these obscure antibodies and their effects on vascular permeability.

3. Idiopathic thrombocytopenic purpura (ITP). Although some investigators have reported and continue to report that the usual cases of ITP have in their serum a platelet agglutinin and that this agglutinin is of diagnostic and prognostic value, our studies on 30 ITP patients so far indicate that the incidence of circulating platelet agglutinin in ITP is not more frequent than it is in any group of patients who have received transfusions, that the presence of platelet agglutinins per se has no bearing on the level of the platelet

count or response to therapy in ITP, and that even an occasional normal individual may have a platelet agglutinin. We had already shown in drug purpura that thrombocytopenia can occur when the antibody concentration was too low to cause platelet agglutination or complement fixation, the complement fixation test for that particular antibody being ten times more sensitive than platelet agglutination test (see 1958 report). We have continued our attempts to demonstrate an antibody in the usual cases of ITP by complement fixation techniques, so far without success.

4. Establishment of a new syndrome. Our studies of 2 patients with an unusual form of ITP have permitted us to differentiate their disease from all other types of ITP and to define a new syndrome. Both patients were middle-aged females who had sudden onset of fulminating purpura associated with a complete absence of platelets approximately 6 days after being transfused during an operative procedure (gastrectomy and lysis of stenosed mitral valve respectively). Both were found to have a plasma antibody which fixed complement with, agglutinated, and lysed all normal platelets and inhibited clot retraction of normal blood. Both patients manifested severe hypotensive reactions to normal blood administered during the height of the disease.

Because hemorrhagic manifestations in one patient were life threatening and steroid therapy was ineffective, splenectomy (effective treatment in usual cases of ITP) had to be considered. However, with evidence that platelets were being destroyed in the circulation by a complement-fixing antibody, it was decided that splenectomy would not be beneficial, but that removal of antibody by exchange transfusion might effect more rapid recovery providing the unknown stimulus for antibody was a transient one. Therapeutic results of a 90% exchange transfusion were better than anticipated. Hemorrhage stopped completely before the exchange was over, platelets rose rapidly after the exchange to normal levels within 2 days and the patient remained well. During the exchange more antibody was removed than could be accounted for by dilution alone, and this along with a fall in plasma complement and a rebound of antibody titer during the first post-treatment day suggested that treatment was unusually effective because antibody had also been sequestered *in vivo* after attachment to transfused platelets.

The second patient had less severe purpura and an initial antibody level approximately 1/10 that of the first patient. She was managed conservatively without splenectomy, and over a period of three weeks her antibody disappeared and platelets returned to normal.

These patients were not only unique in having a complement-fixing, non-drug-dependent anti-platlet antibody (which has never been described before) and the same unusual clinical manifestations but also in showing a remarkable peculiarity after recovery. The platelets which returned in both patients would not react with the antibody of either case in spite of the fact that all normal human platelets (30 different individuals so far) and the platelets from 10 different animals react with the antibodies.

Further studies have been aimed at trying to differentiate the two major possibilities that the "recovery" platelets are coated with some substance (blocking antibody or otherwise) which prevents attachment of antibody or that a somatic mutation has changed the antigenic properties of the platelets. We have not yet been able to prove the former possibility, but the latter possibility seems unlikely in view of the fact that megakaryocytes were plentiful when thrombocytopenia was most severe.

Continued work on this unusual syndrome promises to add to our knowledge of auto-immunity.

5. Observation on an unusual instance of idiopathic cold hemoglobinuria. A child who had the presenting symptoms of dark urine after exposure to cold was found to have a Donath Landsteiner Hemolysin (DLH) with no other associated disease process. This hemolysin disappeared gradually over a one-month period after which a high-titer cold agglutinin developed and subsequently declined. These observations are of interest because there is only one other reported case of idiopathic DLH in a child and no previous documentation of the decay rate of DLH or its being followed by development of a high titer cold agglutinin. Information of this type may help clarify the nature of the DLH and cold agglutinin, two examples of clear-cut auto-antibodies which are completely obscure as to etiology and physiologic significance.

Significance to NIAMD Research. The studies of drug-dependent and idiopathic complement-fixing antibodies have led to a clearer understanding of the basic immunoreactions which result in cellular destruction in vivo and have provided explicit information concerning the significant factors which cause cellular damage in diseases of sensitivity. These studies have numerous implications in the general field of immunology and have bearing on a large group of diseases of suspected sensitivity (e.g., rheumatoid arthritis, collagen diseases, nephritis, etc.) in general medicine.

The finding for the first time that ITP can be caused by a complement-fixing antibody is of special significance because up to now there has been no proof that ITP is an immunologic disease. The establishment of a new thrombocytopenic syndrome will help to clarify the pathogenesis of an obscure group of diseases and provide a rationale for further experimental approaches to effective therapy.

Proposed Course of Project: Further studies of the biochemistry, immunochemistry, kinetics, and physiological significance of immune-reactions which are clinically significant will be continued along lines indicated in the present report.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Shulman, N. R., Clinical implications of a quantitative study of the *in vitro* and *in vivo* reactions of an antibody responsible for thrombocytopenic purpura. Il Pensiero Scientifico, in press.

2. Carpenter, H. M., Jondon, D. J., Shulman, N. R., Tureman, J.R., Toxicology of a Triaryl Phosphate Oil. I. Experimental Toxicology. A.M A. Arch. Indust. Health, 20:234 (1959).

1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Thyroidal Iodoproteins

Principal Investigator: J. E. Ball, M. D.

Other Investigators: Jacob Robbins, M.D., H. Standaert and D. Jermany

Cooperating Units:

Man Years (calendar year 1959):
Total: 3 1/2
Professional: 3
Other: 1/2

Patient Days (calendar year 1959):

Project Description:

Work has continued on the identification and characterization of a particulate iodoprotein. This protein which can be found in relatively small amount in normal thyroid tissue is unusually abundant in certain transplantable rat thyroid tumors. It has now been shown that this particulate iodoprotein can be isolated by a new technique of differential centrifugation in three fractions. In this technique the swinging bucket rotor is used and a small amount of homogenate in 0.88M sucrose is layered onto a large volume of 0.92M sucrose. Multiple spins are made and the sediments collected and assayed. Utilization of this technique for isolation of uricose containing particles of a rat liver homogenate, for example, gave a calculated particle size of from 0.068-0.20 microns. This compares well with values obtained from electron microscopy by Ruff et al, of 0.05-0.25 microns.

Further studies in the isolated particulate iodoprotein were done after solubilization by trypsin, purification by salt extraction and chromatography on diethylaminoethyl cellulose. The protein isolated was hydrolyzed and amino acid analyses performed. An excess of glutamic and aspartic acids was found and histidine and arginine could not be identified. Sedimentation in the preparation ultracentrifuge and measurement of I^{131} showed the solubilized particulate iodoprotein to be of relatively small size ($S_{20,w} < 4S$).

Part B included Yes [ ] No [x]

Preliminary experiments with normal beef thyroid have shown an approximate correspondence between the iodine content of the isolated particulate fractions and their iodinating ability when incubated with I^{131}. If confirmed, this suggests that the enzyme(s) concerned with iodination may iodinate themselves.

Project No. [illegible]
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Serum Thyroxine Binding Proteins

Principal Investigator: Jacob Robbins, M.D.

Other Investigators: J. E. Rall, M.D., W. Merritt and D. Janney

Cooperating Units: Dr. B. S. Blumberg, ARD/NIAMD (Serial No. 1226)
and Dr. W. Beierwaltes, Univ. of Michigan.

Man Years (calendar year 1959): Patient Days (calendar year 1959):
Total: 3
Professional: 2
Other: 1

Project Description:

There has been further investigation of the techniques utilized for identification of thyroxine binding proteins. It has been shown that with ammonium carbonate, sodium bicarbonate, ammediol and borate buffers between pH's of 8-9, electrophoresis reveals in addition to an inter-alpha thyroxine binding protein and albumin, a prealbumin protein which binds substantial quantities of thyroxine. In ammonium carbonate a preliminary estimate shows that the thyroxine binding capacity of prealbumin is about 1.50 µg of thyroxine per ml of serum. In cooperation with Dr. B. S. Blumberg, studies of thyroxine binding proteins in starch gel electrophoresis has been performed. See his report for details. A collaborative project investigated by two-dimension electrophoresis on paper and starch gel the correspondence between the various proteins.

Further work has been done utilizing dialysis systems for estimation of thyroxine binding. If protein is present on both sides of a dialysis membrane, thyroxine will dialyze relatively readily. Utilizing this technique, the effect of various buffer ions has been studied. It has been shown that in the presence of serum, barbital markedly increases the rate of dialysis of thyroxine. Trihydroxy amino methane, borate and phosphate do not show this effect at a similar pH. This strongly suggests that barbital interfere with the association of thyroxine and at least one protein in serum.

Part B included Yes [ ] No [x]

Additional studies have been done in collaboration with Dr. Beierwaltes of the University of Michigan. A family was studied who showed congenital elevation of the inter-alpha thyroxine binding protein. This characteristic was found in the propositus and one of three children. It was associated with entirely normal thyroid function and normal metabolism. However, the serum protein bound iodine was elevated to approximately twice the normal value. Studies of the kinetics of thyroxine disappearance confirmed previous suggestions that the metabolic activity and rate of degradation of thyroxine are governed by the level of free thyroxine in serum.

Serial No. [illegible]
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Studies in Carbohydrate Metabolism

Principal Investigator: Stanton Segal, M. D.

Other Investigators: Alberta Blair

Cooperating Units:

Man Years (Calendar year 1959):
Total: 2
Professional: 1
Other: 1

Project Description:

Various Aspects of Carbohydrate Metabolism Have Been Studied in this laboratory in the past year.

1. Salicylate effects on glucose metabolism. Using the rat diaphragm technique salicyates have been shown to markedly increase the oxidation of C^{14} glucose. At the same time the drug stimulates glycogen breakdown mainly in the early period of incubation. Active muscle phosphorylase was reduced 85 percent. No effect was seen on glucose uptake.

2. Factors affecting galactose metabolism in man. In collaboration with Dr. Yale Topper of the Laboratory of Biochemistry substances affecting galactose metabolism in vitro were studies in normal man and galactosemic children. The hormone progesterone has been found to stimulate galactose metabolism in galactosemic children. Ethyl alcohol has an inhibitory effect on galactose metabolism in the normal subject.

3. Pathways of glucose metabolism in man. Studying singly C^{14} labeled glucose and their rates of conversion to $C^{14}O_2$ has enabled us to construct a biological model of glucose metabolism from which the amount of glucose being metabolized

Part B included Yes [X] No [ ]

by the various pathways may be estimated. Our calculations show that about 10% of overall glucose metabolism is carried out via the pentose phosphate pathway of glucose metabolism.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Segal, S., & Alberta Blair. Effect of salicylate on muscle phosphorylase. Nature 182, 1609, 1958.

Peach, L. A., Segal, S., & V. J. Topper. Progesterone effects on galactose metabolism in pre-pubertal patients with congenital galactosemia and in rats maintained on a high galactose diet. J. Clin. Invest. In press Jan. 1960.

Blair, A., & S. Segal. The isolation of blood glucose as potassium gluconate. J. Lab and Clin. Med. In press June 1960.

Serial No. NIAMD-144C
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Isolated Thyroid Cells

Principal Investigator: Ira Pastan, M. D.

Other Investigator: None

Cooperating Units:

Man Years (Calendar year 1959):
Total: 1/2
Professional: 1/2
Other:

Project Description:

A study of the function of calf and sheep thyroid glands has been undertaken. The glands are treated by mechanical methods and by trypsin so that dispersion of the tissue into single cells takes place. These cells possess the ability to form iodoproteins. The character of these iodoproteins is now under investigation.

In collaboration with Dr. James B. Field a study of the hexose monophosphate pathway in the thyroid gland is in progress. This pathway is under control of thyroid stimulating hormone.

Part B included Yes [X] No [ ]

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Field, J. B., Johnson, P., Herring, B., and Pastan, I. M. In Vitro Stimulation of the Hexose Monophosphate Pathway in Thyroid by Thyroid Stimulating Hormone. (In press).

Serial No. [illegible]
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Humoral Antagonists to Insulin

Principal Investigator: James B. Field, M. D.

Other Investigators: Phyllis Johnson and Betty Herring

Cooperating Units:

Man Years (Calendar year 1959):
Total: 2 1/3
Professional: 1
Other: 1 1/3

Project Description:

The technique utilizing the glucose uptake by the isolated rat hemidiaphragm as a measure of insulin activity has been further modified so that a significant effect can be obtained with as little as 2×10^{-5} units of insulin. Using this procedure extensive studies were done on an insulin-resistant patient who received 38,000 units of insulin/day. In this patient it was possible to demonstrate a high concentration of insulin in her plasma three months after her last known insulin injection. The insulin was identified on the basis of its in vitro stimulation of glucose uptake and glycogen deposition by the rat hemidiaphragm and the abolition of this effect in the presence of insulin antibody. From a measure of the disappearance rates of insulin-I^{131} it was concluded that the insulin was endogenous in origin. When plasma was fractionated by starch block electrophoresis, insulin was found in alpha globulin and between the β and α globulins. Adipose tissue from this patient appeared to be less responsive to insulin than adipose tissue obtained from a normal person and several other diabetic patients.

Several more patients with chronic insulin resistance were studied. Insulin antagonist was demonstrated in all of them and two were subsequently treated with steroids. In one there was no change in the insulin requirement while in the other there was a dramatic decrease. Using the most recent modifications of the rat hemidiaphragm technique it was possible to demonstrate a circulating insulin

Part B included Yes [X] No [ ]

antagonist in some patients with chronic insulin resistance when previous, less sensitive methods failed to detect an antagonist. Several more patients with acromegaly and diabetes were studied, but evidence for an insulin antagonist was found in only one.

Preliminary studies with the rat diaphragm technique suggest that the fasting plasma insulin like activity in the normal is approximately 1×10^{-5} units, a value somewhat lower than previously reported by others.

Studies have also been initiated on the pathways of carbohydrate metabolism in endocrine tissue. In two pancreatic islet cell adenomas it was possible to demonstrate the existence of the hexose monophosphate pathway. This pathway could also be demonstrated in thyroid, adrenal, testis, ovary and parathyroid glands. In the thyroid it was possible to demonstrate a stimulatory effect of TSH on glucose metabolism, especially the hexose monophosphate pathway.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Studies on the circulating insulin inhibitor found in some diabetic patients exhibiting chronic insulin resistance. Field, J. B. and Woodson, M. L. J. Clin. Invest. 38: 551. 1959.

On the Nature of the Metabolic Defect(s) in Diabetes. Field, J. B. Am. J. Med. 26: 659. 1959.

Action of prednisone in insulin-resistant diabetes. Oakley, W. G., Field, J. B., Sowton, G. E., Rigby, J. B., and Cunliffe, A. C. Brit. Med. J. 1: 1601, 1959.

Observations Concerning the Diabetes Mellitus Associated with Werner's Syndrome. Field, J. B. Metabolism, in press

In vitro stimulation of the hexose monophosphate pathway in thyroid by thyroid stimulating hormone. Field, J. B., Johnson, E., Herring, B. and Pastan, I. Biochem. and Biophysical Research Communications. In press.

Serial No. CTAMD-1460
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Human Leucocyte Carbohydrate Metabolism

Principal Investigator: Arnold N. Weinberg, M. D.

Other Investigators: Betty Herring

Cooperating Units:

Man Years (Calendar year 1959):
Total: 1 1/3
Professional: 1
Other: 1/3

Project Description:

Human leucocytes, obtained by venipuncture and sedimentation of the red blood cells with fibrinogen, were incubated in vitro with various plain C^{14} labeled sugars and hormones. Measurement of glucose uptake, galactose uptake, production of lactic acid, and of $C^{14}O_2$ were done in normal controls, diabetics, galactosemics, parents and siblings of galactosemics, and patients being treated with corticosteroids. Also, insulin responsiveness of leucocytes was measured by determining the stimulation of glucose uptake in normals, diabetics and patients on steroids.

Results of these studies to date indicate:

1) Human leucocytes from normal and diabetic subjects actively assimilate glucose, and this process is markedly stimulated by insulin in concentrations as low as 0.1 µ/ml of incubation medium. There appears to be no increased production of $C^{14}O_2$ or of lactic acid coincident with the effect on glucose uptake.

2) In congenital galactosemia there is virtually no oxidation of galactose-1-C^{14} to $C^{14}O_2$, compared with a very active metabolism of galactose by leucocytes from normals. Thus the enzymatic defect previously documented in liver, kidney, small intestine, lens tissue and red blood cells, also can be demonstrated using human leucocytes.

Part B included Yes [ ] No [X]

A test has been devised which can be easily done utilizing either whole blood or leucocytes incubated with galactose-1-C^{14}. To date we have used this test on 5 galactosemic patients with strikingly positive findings, whereas 8 normals and 2 infants with milk allergy were entirely normal.

3) Attempts are in progress to attempt to identify the carrier state (heterozygote) in galactosemia, by utilizing the above test with whole blood and leucocytes from the "normal" parents and siblings of galactosemic children. To date, in 2 of 3 families studied we have found a significant decrease in galactose oxidation in both parents, and in a sibling in one family, which a sibling in the 3rd family was found to be perfectly normal. These studies are being extended at present.

4) Finally, we have been interested in the influence of corticosteroids and growth hormone, *in vivo* and *in vitro*, on the *in vitro* insulin stimulation of glucose uptake in white cells. Results to date suggest some effect on this stimulation, but further work needs to be done to be sure of the significance of the changes.

Serial No. NIAMD-1413
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Studies of Labeled Protein Metabolism and of Thyroid Physiology

Principal Investigator: Charles C. Lewallen, M. D.

Other Investigators: Louis Bunce

Cooperating Units:

Man Years (Calendar year 1959):
Total: 2
Professional: 1
Other: 1

Project Description:

Methods: Sterile, pyrogen free, electrophoretically homogeneous serum albumin is prepared by preparative electrophoresis. The protein is labeled with I^{131} and biological tracer experiments performed in patients and in experimental animals under conditions of varied endocrine status.

NaI^{131} is injected intravenously in subjects of varied thyroid status and serial determinations of thyroid, plasma, and excreted radioactivity are performed.

Conditions effecting the iodination yield and the distribution of label in I^{131} iodoalbumin have been investigated--the latter by paper chromatographic and high voltage electrophoretic analysis of enzymic digits of the iodoalbumin.

Results: By a modification of MacFarlane's jet iodination method (1) it has been possible to prepare an iodoalbumin in good yield in which more than 95% of the organically bound iodine occurs as monoiodotyrosine. Biologically this preparation shows a metabolic half life of 18-21 days and less than 2% of rapidly

(1) MacFarlane, A. S. - Labelling of plasma proteins with radioactive iodine. Biochem. J. 62: 135, 1956.

Part B included Yes [X] No [ ]

degraded components. In the course of investigation of the products of enzymic digestion of this preparation by paper chromatography, an inconstant interesting chromatographic "artifact" was observed in a butanol ammonia system in which monoiodotyrosine migrated as 2 distinct zones, one with an Rf of about .10 and one with an Rf of about 0.05. These zones when eluted showed identical behavior in other chromatographic systems and when subjected to high voltage electrophoresis. Since this phenomenon has considerable bearing on the problem of identification of the amino acid products of digested iodoalbumin it has been investigated in some detail employing I^{131} labeled monoiodotyrosine. The double zoning could not be regularly reproduced by any of the following procedures:

(1) Variation of temperature during chromatography of a degree compatible with ambient temperature fluctuation.

(2) Variation in pH of the starting zone from 2-12.

(3) Salt loads in the starting zones as high as 100 micrograms.

(4) Inclusion in the starting zone of anionic detergents, cationic detergents, or phenol.

(5) Variation of distance of the starting zone from the end of the paper.

(6) Variation of conditioning time.

(7) Variation of ammonia concentration in the developer.

(8) Impregnation of the paper with heavy metal cations.

(9) Oversaturation of the developing solvent with aqueous ammonia to the extent of separating phases in the developing solvent.

It has been possible to reproduce the phenomenon with regularity at constant temperature regulation incorporating certain metallic cations in the starting zone provided the molar cation monoiodotyrosine ratio, the concentration of ammonia in the developer, and the conditioning time are properly adjusted. If the mechanism of the phenomenon can be clarified, it may shed some light on the not infrequent occurrence of "unknowns" in the chromatographic analysis of biological materials containing iodinated compounds.

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Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Lewallen, C. G., Berman, M., and Rall, J. E.: Studies of iodoalbumin metabolism. I. A mathematical approach to the kinetics. Journ. of Clin. Invest. 38: 66, 1959.

Lewallen, C. G., Rall, J. E., and Berman, M: Studies of iodoalbumin II. The effects of thyroid hormone. J. Clin. Invest. 38: 88, 1959.

Serial No [illegible]
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: The Physical Chemistry of Proteins

Principal Investigator: Harold Edelhoch, Ph.D.

Other Investigators: Henry Metzger, M. D., Roland Lippoldt

Cooperating Units:

Man Years (Calendar year 1959):
Total: 2 1/2
Professional: 1 1/2
Other: 1

Project Description:

A procedure based on differential centrifugation (in the Spinco Model L) has permitted the purification of calf thyroglobulin such that only a single symmetrical boundary is observed on sedimentation of a 1.5% solution. The light scattering molecular weight of this preparation was in close agreement with the sedimentation-diffusion value, which provides another criterion of its molecular uniformity.

The denaturation of thyroglobulin has been studied by both kinetic and molecular methods as a function of pH and temperature.

At all pH values observed the denaturation of thyroglobulin, as followed by its insolubility near its isoelectric point, obeys first order kinetics. In the neutral pH zone (7 to 9) the rate varies slowly with pH ($[H]^{-0.35}$) whereas it changes rapidly with temperature, i.e., the activation energy is 160,000 kcals/mole. However about pH 11, the rate increases very fast with pH--about 10-fold greater than at neutral pH values--while the temperature coefficient is only about one half that observed in the neutral range.

Part B included Yes [X] No [ ]

In order to facilitate interpretation of the kinetic data it is useful to know the molecular configurational changes that occur on denaturation. We have therefore performed sedimentation, viscosity, turbidity and optical rotatory measurements on solutions of thyroglobulin which have been heated to various temperatures--at several pH values.

In an earlier study on the splitting of thyroglobulin into subunits (by alkali) it was postulated that an activation energy barrier governed the rate of dissociation. This hypothesis has now been confirmed by showing that an increase in temperature accelerates the rate (and affects the equilibrium) of dissociation. The dissociation reaction however appears to precede the denaturation. Surprisingly the denaturation phase of the reaction seems to produce very little additional configurational changes above that encountered by heating to a temperature just short of altering the solubility properties of thyroglobulin solutions.

In the neutral pH zone the smallest molecular unit formed from native thyroglobulin (19S) by denaturation has a sedimentation constant of 12S. In alkali (pH >11.5) the denatured molecule has an S value of about 8. It is interesting that both of these molecules appear to behave as globular proteins when examined by sedimentation and viscosity. Optical rotatory data tends to confirm this picture in that the S-12 molecule is formed with practically no change and the S-8 with only a small increase in levorotation.

It would appear therefore that denatured thyroglobulin does not show the molecular unfolding normally observed with protein denaturation. Only chymotrypsinogen shows behavior similar to thyroglobulin in that essentially no change in its macromolecular properties was observed when it was denatured by heat at pH 3.

PHS-NIH
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Edelhoch, H: The Denaturation of Pepsin. IV. The Effects of Temperature. Biophys. Biochim. Acta. In press.

Edelhoch, H: The Properties of Thyroglobulin. I. The Effects of Alkali. J. Biol. Chem. In Press.

Edelhoch, H, and Lippoldt, R. E.: The Properties of Thyroglobulin. II. The Effects of Sodium Dodecyl Sulfate. J. Biol. Chem. In Press.

Rall, J. E., Robbins, J., and Edelhoch, H., Annals New York Acad. Sci. In Press.

DHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Synthesis of Analogs of Thyronine. Synthetic and Mechanistic Studies

Principal Investigator: Hans J. Cahnmann, Ph.D.

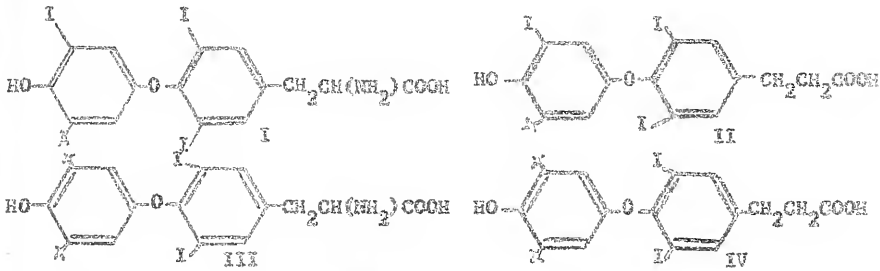
Other Investigators: Annamarie Hofer, Ph.D.

Cooperating Units:

Man Years (Calendar year 1959):
Total: 2
Professional: 2
Other:

Project Description:

Synthesis of partially and completely hindered analogs of thyronine such as (I), (II), (III), (IV):

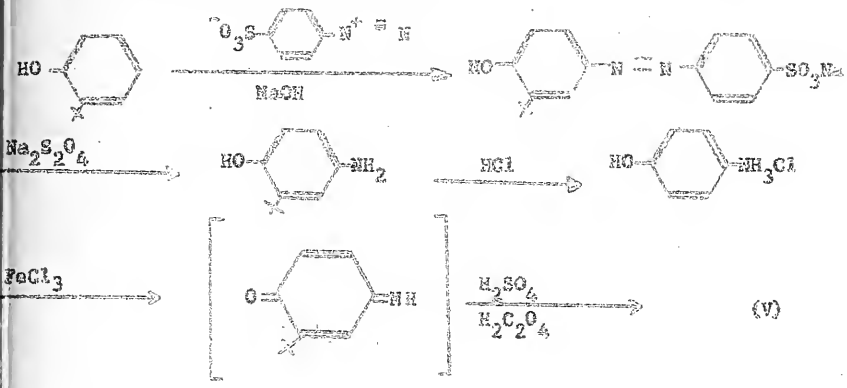


$\frac{t}{}$ = t-butyl

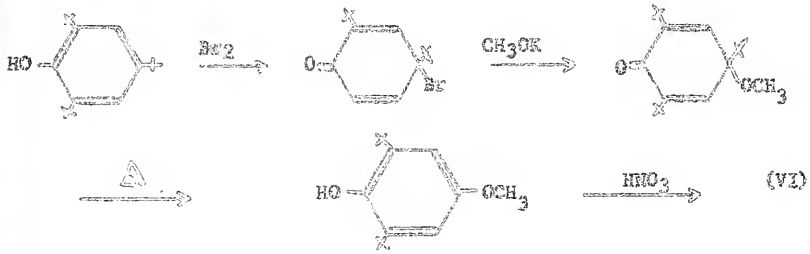
The two quinones (V) and (VI) required as intermediates for the preparation of compounds (I) through (IV) have been synthesized.

Part B included Yes ☐ No ☐

Compound (V), not previously described in the literature, has been synthesized as follows:



Compound (VI) could not be synthesized in an analogous manner due to the presence of a strongly hindered phenol group. It was prepared as follows:



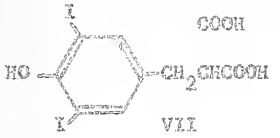
Although this synthesis led to a substance with different physical constants than those described in the literature for (VI), elemental analysis as well as the infrared spectrum indicate that it is the desired quinone. (This may be a case of polymorphism.)

The quinones (V) and (VI) will be reduced to the corresponding hydroquinones. It is hoped that these can be converted to the analogs of (I) through (IV) in a series of reactions analogous to those commonly used in the synthesis of thyroxine and its analogs.

Influence of the structure on the side chain on the nonenzymic conversion of deamino analogs of diiodotyrosine to analogs of thyroxine: It has been found previously that in the nonenzymic incubation of the propionic acid analog of diiodotyrosine the corresponding analog of thyroxine is formed in particularly good yield. In contrast, the 2-methyl and 2-phenylpropionic acid analogs yield only very little or no analog of thyroxine. This raises the question whether this inhibition of the condensing reaction was due to steric or electronic influences. (Methyl and phenyl are bulkier and more electron releasing than hydrogen).

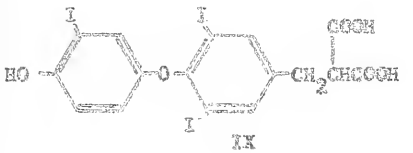
An analog of diiodotyrosine with a propionic acid side chain in which an electron attracting group, viz.-COOH, is attached to the carbon atom 2 of the propionic acid side chain was therefore incubated. Only a very small amount of the corresponding analog of thyroxine was formed which indicates that the inhibition of the condensing reaction by the methyl and phenyl groups is not due to the electron releasing properties of these substituents.

Synthesis of the starting material (VII) for the foregoing experiment and of several new analogs of thyroxine (VIII), (IX), (X):

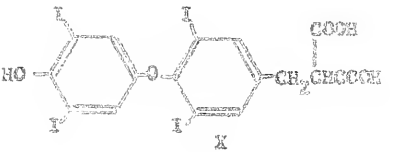


VII

VIII

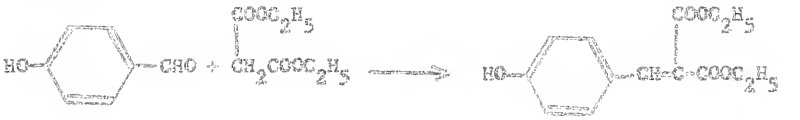


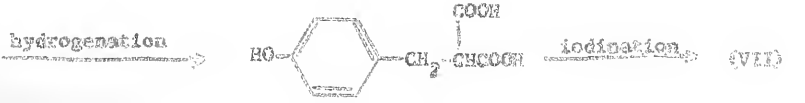
IX



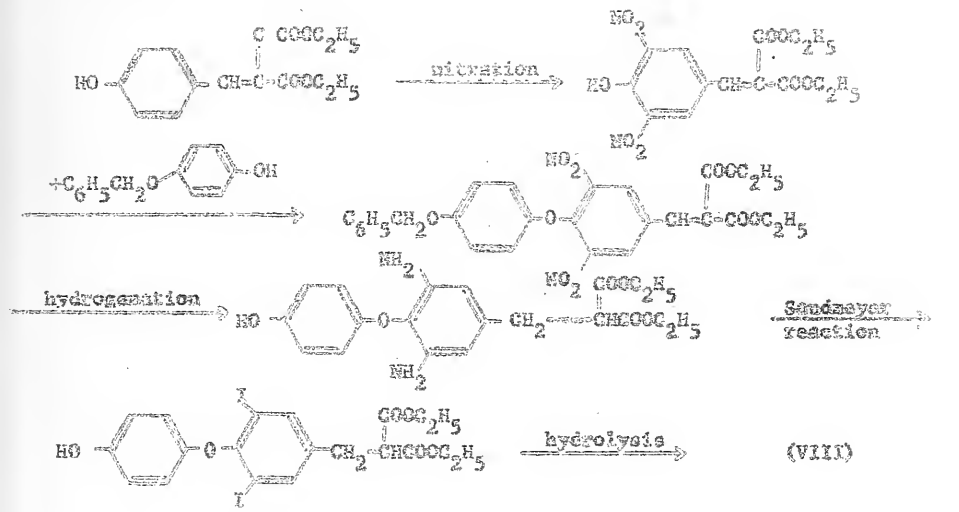
X

Substance (VII), not described in the literature, was prepared as follows:



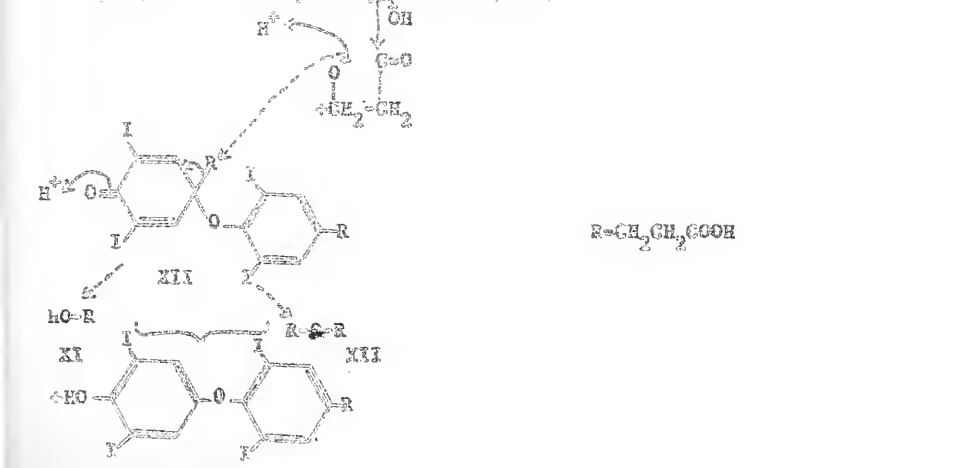


Substance (VIII) was synthesized as follows:



Substances (IX) and (X) were obtained by partial or complete iodination of (VIII). Preliminary bioassays showed that both (IX) and (X) induce metamorphosis in the tadpole.

Mechanism of the elumination of the aliphatic side chain in the nonenzymic incubation of diiodophloretic acid: Further study of this mechanism led to the hypothesis that the propionic acid side chain is eliminated as a cyclic compound (propiolactone) which then reacts further, either with a proton to form hydracrylic acid (XI), or with the quinol ether intermediate (XII) to form dihydracrylic acid (XIII).



Miscellaneous investigations: It has been found that a sodium borohydride reduction of p-hydroxyphenylpyruvic acid to the corresponding lactic acid is possible only under certain experimental conditions (due to keto-enol tautomerism and to the formation of an enol borate complex). Favorable conditions for the reduction have been determined.

It has also been found that p-hydroxyphenylpyruvic acid is rapidly degraded by alkali under mild conditions (0.1 N NaOH, room temp.) to form almost quantitatively p-hydroxybenzaldehyde. Syntheses of this and related keto acids, involving the use of alkali (such as reported in the literature) must therefore be rejected.

Tetraiodothyropyruvic acid also shows keto-enol tautomerism as evidenced by a typical ketone spectrum and the formation of a borate complex.

The photochemical reaction of iodophenols which leads to the elimination and reincorporation of an unknown iodide-like substance [Tata, Bioch. J., 72, 214 (1959)] has been repeated with thyroxine. The phenomenon described by Tata could not be reproduced.

NIH-NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Matsuura, T., & H. J. Cahnmann. Model Reactions for the Biosynthesis of Thyroxine. I. Structural Influence of the Side Chain in Analogs of Diiodotyrosine on their Conversion to Analogs of Thyroxine. J. Am. Chem. Soc., 81, 871 (1959).

Cahnmann, H. J. & T. Matsuura. Model Reactions for the Biosynthesis of Thyroxine. II. The Fate of the Aliphatic Side Chain on the Conversion of 3,5-Diiodophloretic Acid to 3,5,3',5'-Tetraiodothyropropionic Acid. J. Am. Chem. Soc., 82, (1959).

Matsuura, T., & H. J. Cahnmann. Model Reactions for the Biosynthesis of Thyroxine. III. The Synthesis of Hindered Quinol Ethers and their Conversion to Hindered Analogs of Thyroxine. J. Am. Chem. Soc., 82, (1959).

Serial No. NIAMD-19GC
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Studies on the Mechanism of the Hepatic Capture of Insulin

Principal Investigator: Glenn E. Mortimore, M. D.

Other Investigators: Frank Tietze, Ph.D., Nancita Lomax

Cooperating Units: Intermediary Metabolism Section, Laboratory of Biochemistry and Metabolism, NIAMD-26

Man Years (Calendar year 1959):
Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: In studies previously reported by the above investigators, it was shown that lightly iodinated insulin is rapidly removed from circulation by the intact, cyclically perfused liver. Since iodoinculin appeared to be concentrated by the liver prior to its degradation, a separate step, occuring before insulin proteolysis, was postulated. The aim of this study has been to elucidate the mechanism of this step or steps.

Methods: 1) Cyclic perfusion of rat liver with oxygenated blood or plasma. 2) Partition and identification of degradation products by trichloracetic acid precipitation and paper chromatography. 3) Radioactivity assay.

Major Findings: In an attempt to inhibit selectively the process of insulin degradation, and thus "isolate" an initial concentrating mechanism, a series of liver perfusions were carried out at about 3°C. Such studies revealed that, whereas iodoinsulin was removed from circulation and concentrated by liver tissue, its subsequent, degradation was completely abolished. Virtually all of the iodoinsulin radioactivity removed from the perfusate could be sedimented after centrifuging the homogenized liver.

Part B included Yes [ ] No [X]

thus demonstrating its binding to a particulate fraction of the liver cell. Crystalline insulin, but not ACTH, prolactin, or growth hormone, competed with iodoinsulin for the site of binding Iodoinsulin, rendered biologically inactive by alkali treatment, was not bound.

Since control experiments indicated that the liver retained appreciable proteolytic activity at 0°, our failure to observe degradation by the perfused intact liver suggested the presence of a block, functionally interposed between the process of binding and degradation. Further evidence supporting the existence of an intermediate step emerged from a series of experiments with EDTA (Versene). This compound was shown to cause a substantial reduction in the rate of iodoinsulin degradation by the perfused, intact liver. However, no effect was observed on the binding of iodoinsulin or its degradation in liver homogenates. It is tempting to speculate on the nature of this intermediate process. Certainly this data are in accord with some published reports concerning the deleterious effects of cooling and Versene on membrane function.

A comparison of the intact liver and the homogenate with respect to the ability of ACTH and glucagon to inhibit iodoinsulin degradation revealed significant differences. Neither peptides inhibited insulin degradation in the intact liver, yet both caused inhibition in homogenate experiments. When the degradation of heavily iodinated insulin and alkali-treated iodoinsulin were each compared in the two systems, the extent of proteolysis by the intact liver was far less.

Significance to NIAMD Research: The eventual fate of insulin in its reaction with the intact liver cell is its destruction by proteolysis. The results of the above studies indicate, however, that its access to the proteolytic enzymes involved is not one of passive diffusion through a semipermeable membrane, but may entail a series of more complicated steps. The finding that insulin is bound initially and selectively, suggests a mechanism whereby certain cells may sequester insulin rapidly. Whether mechanisms elucidated here will eventually find counterparts in insulin-responsive cells, such as muscle and adipose tissue, is a question of considerable importance.

Proposed Course: See Cytological Localization of Insulin.

Serial No. [illegible]
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Cytological Localization of Insulin

Principal Investigator: Glenn E. Mortimore, M. D.

Other Investigators: Frank Tietze, Ph.D., George Glenner, M. D.
M. W. Emmert, Ph.D., Nancita Lomax

Cooperating Units: 1) Intermediary Metabolism Section, Laboratory of Biochemistry & Metabolism 2) Section on Histochemistry, Laboratory of Pathology and Histochemistry 3) Section of Biochemistry of Amino Acids, Laboratory of Pharmacology and Toxicology, NIAMD-26,73,102

Man Years (Calendar year 1959):
Total: 3 3/4
Professional: 3
Other: 3/4

Project Description:

Objectives: To identify the cellular site or sites of insulin binding. Should technics prove satisfactory, an attempt will be made to correlate the metabolic responses to insulin with its cytological location.

Methods: 1) Preparation of isolated cell suspensions. 2) Organ perfusion. 3) Paper electrophoresis. 4) Frozen tissue sectioning. 5) Fluorescence microscopy. 6) Radioautography.

Major Findings: Preliminary I^{131} radioautographs were made on sections of liver perfused with iodoinsulin at 3°C. Although the distribution of radioactivity was fairly uniform, there appeared to be some localization along the sinusoidal borders. There was no heavy concentration of radioactivity adjacent to the Kupffer cells and it may be concluded that the

Part B included Yes [X] No [ ]

bulk of iodoinsulin was bound in the vicinity of the parenchymal cell cords.

To improve cytological resolution a series of experiments, employing a fluorescing derivative of insulin, visualizable under fluorescence microscopy, has been started. Crystalline insulin was reacted with fluorescein isothiocyanate, yielding an insulin-fluorescein derivative which is strongly fluorescent and retains hypoglycemic activity. Thus far we have been successful in visualizing fluorescence bound to isolated liver cells in suspension. Since adequate controls have not yet been completed, no conclusions can be drawn with reference to the sites of specific binding.

Significance to NIAMD Research: A current theory of insulin action is that it in some way accelerates the membrane transfer of glucose. Since the primary aim of this study is to localize the sites of insulin binding and perhaps to visually follow its fate within the cell, information gained in this way might prove useful in strengthening or modifying the above hypothesis. A technic of this kind might be applied to other protein or peptides as well.

Proposed Course: To continue the work as outlined above, eventually extending it if possible, to insulin responsive cells.

[illegible]
Individual Project Report
Calendar Year 1959

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Mortimore, G. E. and Frank Tietze. Studies on the Fate of Insulin-I^{131} in the Perfused Rat Liver. Metabolism 8: 479 (1959).

Mortimore, G. E. and F. Tietze. Studies on the Mechanism of Capture and Degradation of Insulin-I^{131} by the Cyclically Perfused Rat Liver. Ann. N. Y. Acad. Sci. 82: 329 (1959).

PHS-NIH
Individual Project Report
Calendar Year 1959

Part A:

Project Title: Binding of Cortisol by Serum Protein

Principal Investigator: Saul W. Rosen, Ph.D., M. D.

Other Investigator: Ruth Johnson

Cooperating Units:

Man Years (Calendar year 1959):
Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

Experiments are in progress on the attempted isolation of the corticosteroid binding globulin(s) of human serum. Using tritium labelled cortisol and the technique of protein chromatography on anion exchange cellulose (Sober and Peterson), it has been found that the corticosteroid binding globulin(s) can be eluted over a relatively narrow range of pH and ionic strength. Most of the corticosteroid binding activity has been located in Cohn fraction IV-4, whose titer of the specific globulin(s) is 5-10x that of serum. Further fractionation of column eluates and of IV-4 is in progress and attempts are in progress to isolate and characterize a discrete binding species by preparative starch gel electrophoresis.

It has been shown (Daughaday, Sandberg and Slaunwhite) that protein-bound cortisol migrates anodally at a pH where albumin is isoelectric. They have suggested the likelihood, as a consequence that the binding globulin is a glycoprotein. Experiments in our lab using neuraminidase suggest that neuraminic acid is not involved in cortisol binding. Similar behavior in electrophoresis and towards neuraminidase has been shown by Ingbar for thyroxine binding protein (TBP). The presence of TBP activity in prealbumin and the absence of neuraminic acid in a pure preparation of a prealbumin (Schultze) may be pertinent and will be explored.

Part B included Yes [ ] No [X]

PHS-NIH
Individual Project Report
Calendar Year 1959

Serial No. 159
Office of the Director
Bethesda

Part A

Project Title: Administration

Principal Investigators: Dr. Floyd S. Daft, Director
Dr. G. Donald Whedon, Assistant Director
Mr. W. G. Baylis, Executive Officer

Project Description:

Administration: The programs of the National Institute of Arthritis and Metabolic Diseases encompass three major areas: (1) Basic Laboratory Research; (2) Clinical Investigations; and (3) Extramural Programs (Research Grants, Training Grants, Research Fellowships, and Graduate Medical Training Grants). In addition, the Institute is responsible for providing business management services to the Interdepartmental Committee on Nutrition for National Defense, a world-wide survey activity. The Office of the Director is responsible for planning and directing the overall administration of the Institute, in conducting, fostering and coordinating investigation of the cause, prevention, diagnosis and treatment of arthritis, rheumatism and metabolic diseases; for maintaining effective operating relationships with other Institutes, and with other units of the Public Health Service, with the Department of Health, Education and Welfare, other Governmental Agencies, and public and private organizations carrying on related functions. The Office of the Director also participates in determining policies governing the National Institutes of Health.

The Director, with the cooperation and advice of his staff, sponsored several cooperative conferences in collaboration with the American Rheumatism Association and the Arthritis and Rheumatism Foundation. Chief among these were the Conference on the Comparative Pathology of Arthritis and Rheumatism which was held in Washington, D.C., and the Congress on the Host Response Mechanism in Rheumatoid Arthritis, which was held in Atlantic City, New Jersey. Also, the Office of the Director participated very actively in planning and coordinating the Second Pan American Congress on Rheumatic Diseases, held in Washington, D.C. and Bethesda, Maryland, June 2 through June 6.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

Annual Report - Extramural Programs

January 1, 1959 - December 31, 1959

During the past year this Institute has continued, at an accelerating rate, to foster research, and training for research, in those areas of medical science for which it assumes a prime responsibility. An increased number of research and training grants from the National Institute of Arthritis and Metabolic Diseases were awarded to experienced research teams to support a broad and well coordinated attack on the problems associated with arthritis, diabetes, gastroenterology, physical biology, cystic fibrosis, and various metabolic diseases. As in the past, the diabetes and arthritis programs constituted the major field of emphasis, but increased activity in the other programs has been encouraged as a logical step in rounding out the over-all program of the Institute. As had been anticipated the expanding physical biology program is demonstrating its value, not only in terms of direct contributions, but also in developing an awareness, in the various disciplines, of the desirability of using the techniques of physics and physical chemistry to complement other less precise methods. It is felt that this program is serving to help achieve a more desirable balance between purely clinical and basic research. Closer cooperation between the clinically oriented investigator and his counterpart in the basic disciplines, based on mutual respect and dependence, is thus being promoted by the current program.

Research grant awards made this year supported research in both clinical and basic areas which, as in the past, ranged from investigations of incidence and etiology to treatment and rehabilitation. The various disciplines involved include: physiological and biological chemistry, general metabolism, nutrition, endocrinology, pathology, hematology, pharmacology, embryology, bacteriology, physiology, biophysics, biophysical chemistry, surgery, general medicine, and others to a lesser extent. Budget increases in both training and research have permitted a controlled expansion in all facets of the program and it is anticipated that further expansion will continue as a logical and desirable consequence of the over-all objectives of the Institute program.

It is gratifying to note that as the training program approaches some degree of maturity, a considerable number of former trainees (about 50%) are establishing independent research programs. There appears to be no doubt that the existence of training programs in key institutions throughout the country has had, and will continue to have, a healthy effect in focusing attention on the major research programs of this Institute and in creating an atmosphere conducive to academic medicine.

Research Grants Activities

As of December 1959, this Institute was supporting approximately 1640 research projects by means of grants having a gross annual total of about $25,568,000. The average grant was thus $15,500. These grants are distributed among a total of 205 institutions located in 42 states, the District of Columbia, Puerto Rico, and six foreign countries. They support both clinical and basic research related to arthritis, various metabolic diseases including diabetes and thyroid disease, liver diseases, nutritional diseases, cystic fibrosis, kidney diseases, gastroenterology, and also research in physical biology.

The National Advisory Arthritis and Metabolic Diseases council, at its three meetings in the calendar year 1959 reviewed 1368 research grant applications having a total requested amount of $23,783,203. Of these, 809 were recommended for approval in the amount of $12,257,657. The approved applications consisted of 138 competitive continuations in the amount of $2,292,043; - 576 new applications in the amount of $8,989,361; - and 95 supplemental requests totaling $976,253. Total council actions relative to review and approval for the year are summarized in tabular form below.

Research Grants

	Requests		Approvals	
Council Meeting	No.	Amount	No.	Amount
March 1959	434	$ 6,969,634	296	$ 4,291,338
June 1959	932	13,310,786	932	13,310,786
	562	10,279,679	440	6,950,622
Nov. 1959	372	6,533,890	257	3,786,088
Total	1,368[1]	$23,783,203[1]	1,925[2]	$28,338,834[2]
			- 184[3]	-2,770,392[3]
		Total Approvals	1,741	$25,568,442

1/ Excludes 932 requests for reaffirmation of previously recommended support in the amount of $13,310,786.

2/ Includes 932 requests for reaffirmation of previously recommended support in the amount of $13,310,786.

3/ Applications which were approved in March 1959 or November 1958, but which were not paid because of a lack of funds, were reconsidered at the June 1959 meeting. These figures are subtracted to obtain a true total because they appear twice in the body of the table.

A number of examples, selected at random from approximately 1640 active research projects supported during the past year, serve to illustrate the nature and variety of this complex program. All are related directly or indirectly to problems which lie within the categorical interests of this Institute.

In the field of diabetes, the biochemistry of insulin in vivo continues to be the subject of intensive research. Dr. Robert H. Williams and co-workers confirmed the fact that insulin is rapidly degraded by virtually all body tissues. The mechanism of the degradation is not fully understood as yet but the studies indicate that it occurs in at least three ways; i.e., enzymatic and non-enzymatic reduction and by proteolysis. Glucagon, by way of contrast, appears to be degraded only by proteolysis. A better understanding of these processes might serve to explain the wide variance of insulin requirements among diabetics. The search for effective oral antidiabetic drugs continues along with research as to the mode of action of those that have already enjoyed some success. It has been suggested that carbutamide and tolbutamide stimulate release of insulin from the pancreas, thus promoting hypoglycemia. However, other evidence shows that this is not their only mode of action although it is generally agreed that small quantities of insulin (endogenous or exogenous) are required to make these drugs effective. Dr. Piero P. Foa and co-workers reported that experiments on a similar drug, chlorpropamide, indicated that its hypoglycemic effect is due, at least in part, to a decreased liver glucose production and offered no evidence of a pancreatropic action. They did not ascertain whether insulin was necessary to the hypoglycemic effect of chlorpropamide. One of the newer oral drugs under investigation is phenethyldiguanide, called DBI, PEDG, or PEBG. This drug is independent of insulin. Williams, Foa, and others have variously reported that it inhibits succinic dehydrogenase and cytochrome oxidase, thus leading to tissue anoxia and inhibition of oxidative phosphorylation. As a consequence, products of the Krebs tricarboxylic cycle accumulate. DBI is believed to inhibit gluconeogenesis and to stimulate anaerobic glycolysis. In spite of its rather severe toxicity, this drug may be of considerable value in the treatment of diabetes when used in small doses in conjunction with insulin. Dr. Foa, in reviewing experimental evidence relative to the sulfonylureas, states that these drugs apparently act by suppressing liver glucose production, but only when insulin is injected or released in "permissive" amounts. He cautions that long therapy with such drugs should be attempted with caution, since the suppression of hepatic glucose may be a sign of liver injury.

Dr. Stefan S. Fajans has presented evidence indicating that some of the oral antidiabetic drugs may have an important use in the prevention of severe diabetes in persons having asymptomatic diabetes. Prolonged administration of tolbutamide in such cases brought about an improvement in the glucose tolerance test, even though the drug was withheld for two days before the test.

Dr. Arnold Lazarow reported that electronmicroscopy studies on renal biopsies from human diabetic subjects early in the course of the disease reveal a significant thickening of the glomerular basement membrane. This is observable prior to the appearance of clinical symptoms of the complications of diabetes. The same investigator has found that sub-diabetes in the pregnant rat produces a statistically significant increase in the virth weight of the fetus and a three-fold increase in fetal mortality. These abnormalities in the experimental animal are similar to those reported for the prediabetic state in man. Dr. David Harker and co-workers have devised a technique for introducing heavy elements into protein molecules, without seriously damaging them. This permits the use of X-ray diffraction techniques to study the atomic arrangement within the molecule. An understanding of the structure of the molecule leads to a better understanding of the nature of, and reasons for, the highly specific reactions that are characteristic of protein molecules in general. Drs. Truman S. Licht, Milton Stern, Harry Shwachman, and Andre J. deBethune report the perfection of techniques for use in the diagnosis of cystic fibrosis. Making use of the fact that the sweat of cystic fibrosis patients has an elevated sodium chloride content, these investigators have reported two rapid, sensitive, and accurate methods for determining the salt content of sweat. In one, the pCl is measured potientiometrically, while in the other the electrical conductivity of diluted sweat is determined. On the average there is from two to three times more sodium chloride in sweat from cystic fibrosis patients than from normals. Dr. Zacharias Dische reported the development of a method for fractionating the fuco-mucoids of the urine of children with cystic fibrosis. The method makes use of organic solvents and continuous flow electrophoresis. The above examples illustrate the use of physical and physico-chemical methods in medical research problems. The NIAMD co-sponsored (with NIAID) an international congress on cystic fibrosis in January 1959. The conference was attended by seventy leading investigators and was designed to suggest and stimulate new research concerning the basic nature of cystic fibrosis. It included discussions of possible new avenues of approach to prevention and treatment.

Various pathological conditions, including cirrhosis of the liver, gastrointestinal hemorrhage, acute hepatic failure due to viral hepatitis, and the ingestion of liver toxins, lead to dangerously high blood ammonia levels because of the inability of the liver to detoxify ammonia produced in the GI tract. Drs. J. S. Najarian, H. A. Harper, and H. J. MacCordle have found that the intravenous injection of arginine reduces blood ammonia by increasing the production of urea, an ammonia-containing compound which is excreted in the urine. Dr. J. K. Isley and co-workers have developed a technique for measuring absorption from the colon using radioactive sodium iodide. In a group of 5 patients with ulcerative colitis, 1.6% of the sodium iodide was absorbed in 15 minutes, while 6.0% was absorbed from the colon of 14 normal individuals. The technique promises to be useful in evaluating the condition of the colonic mucosa.

Non-tropical sprue is a disease associated with long-lasting diarrhea, weakness, and weight loss resulting from failure to absorb certain proteins properly. Drs. M. H. Sleisenger, T. P. Almy, and others have shown that a diet completely free of gluten (cereal protein) provides a ready means of controlling the disease and recommend such treatment without reservation. Further research is being carried on to study the fundamental pathogenetic mechanism which leads to non-tropical sprue. Drs. D. W. Elliott, R. C. Williams, and R. M. Zollinger have been able to show that moderate to fatally severe pancreatitis may be caused by a back flow into the pancreas of a mixture of bile and pancreatic secretion. When the common duct is blocked, as by gallstones, pancreatic secretions may enter the gall bladder where the pancreatic enzyme trypsinogen is converted to trypsin, a powerful proteolytic enzyme. The mixture of bile and pancreatic secretions may then reenter the pancreas where the trypsin can cause severe tissue damage. This is probably the first direct evidence to support a theory which has been held by some investigators. Further work is contemplated in an effort to develop surgical techniques to correct conditions which permit the above sequence of actions to occur. In other metabolic studies Dr. C. W. Vermeulem has shown that in animal experiments a high calcium intake reciprocally reduced urinary phosphorus concentration and actually decreased the incidence of urinary tract stone development. Dr. W. H. Boyce implicates certain unusual muco-proteins in the urine as being important factors in stone formation. Both of these findings tend to allay fears that a high milk intake in adult man might encourage urinary calculi formation.

Research in the field of arthritis continues to center around the rheumatoid factor and the search for more efficacious methods of treatment. A conference on the pathology of arthritis and rheumatism which was co-sponsored by NIAMD was attended by 45 investigators, many of whom were grantees of this Institute. The conference was successful in fostering an exchange of information between veterinarians, pathologists, and clinicians, and in more clearly defining the similarities and dissimilarities between human and animal arthritis. Several new types of the disease were reported for the first time. In studies on the rheumatoid factor, Drs. Robert C. Mellors, Ralph Heimer, Josue Corcos, and Leonhard Korngold demonstrated for the first time that the factor is present in human tissue and, in fact, found evidence as to the site of its formation. It is hoped that further study will make possible a more specific test for preclinical arthritis than is now possible. The rheumatoid factor, according to these investigators, is produced in certain plasma cells and in germinal-center cells.

Drs. W. Roy Slaunwhite, Jr., and Avery A. Sandburg have reported the isolation of a new corticosteroid-binding protein in human plasma which they named transcortin. The protein is an alpha-globulin and like some other plasma proteins is believed to be part of a mechanism for transporting certain hormones in the body. Although it has been known for some time that plasma proteins bind steroids, not many of these

proteins have been isolated and characterized. A new method for determining the reserve capacity of the pituitary to secrete ACTH has been developed by Dr. G. W. Liddle and co-workers. Essentially the test consists of using the agent SU-4885 to inhibit the production of corticol by the adrenal gland and this brings about a compensatory increase in ACTH secretion in patients with a normal pituitary gland. Tests on many patients indicate the reliability of the new technique. Dr. Tsamparlis has studied the effect of Zoxazolamine in 43 gouty patients. In either single doses or after prolonged administration, the drug proved effective in reducing blood uric acid levels and increasing urinary excretion of uric acid. In 16% of the patients undesirable side effects such as nausea, diarrhea, or headache forced discontinuance of the therapy. The same investigator, with Dr. C. McEwen, reports that the use of colchicine as one of the most useful aids in diagnosing gout has lead to an interesting and perhaps important finding that intravenous administration of colchicine apparently benefited several patients with acute non-gouty arthritis. The intravenous mode of administration was found to be far superior to oral administration both as to speed of action and lack of severe side effects. Dr. Herfert and co-workers have reported apparently successful treatment of six arthritics by means of surgery which is referred to as "extended sympathectomy". The operation relieves arthritic pain and facilitates rehabilitation in patients who are willing to cooperate in the prescribed exercise therapy. Dr. C. McEwen and several other investigators have continued studies to establish the nature of the relationship between rheumatoid arthritis and systemic lupus erythematosus. In one group studied, ten relatives of lupus patients had positive tests for rheumatoid factor while three had clinical rheumatoid arthritis. Five relatives, two in the same family, showed hypergammaglobulinemia.

During 1959, more than 1,000 scientific papers were published by investigators who were supported wholly, or in part, by research grant funds from this Institute.

Training Grant Activities

The continued scarcity of qualified young scientists who are interested in careers in academic medicine emphasizes the current value and future potential of the training program. Increases in the budget have permitted a reasonable expansion in this area, but the saturation point is not yet in sight. During the past year a conscious effort has been made to encourage more training for research in the basic sciences in order to better balance this phase of the program with the more clinically oriented training. Thus, several of the older projects have been either modified or eliminated to bring them in line with current policy. Four committees, composed of men who are outstanding teachers and investigators in the traditional areas of responsibility of this Institute, continue to provide guidance in the training program, not only in the review and approval of applications, but also with respect to the over-all aims and policies

of the program. Two meetings of NIAMD training grant program directors were held during the year. About 50 directors from the arthritis and 25 from the gastroenterology programs met to discuss means of improving methods used to attract competent investigators to these fields. Discussions were held to establish uniform thinking regarding how the time of a trainee should be divided between laboratory and clinical training. The necessity of both types of training was agreed upon.

During 1959 there were 168 active training grants which were distributed by category as follows: Arthritis, 43; Diabetes, 53; Gastroenterology, 27; Hematology, 17; Metabolism and Endocrinology, 10; Physical Biology, 10; and other, 8. Comparison of these figures with those of 1958 reveals the relatively greater growth of the newer programs in Gastroenterology and Physical Biology.

1959

Requests		Approvals	
No.	Amount	No.	Amount
153	$ 3,588,370	124	$ 2,141,686
	Previously recommended	152	3,366,746
	Total	276	$ 5,508,432

These figures represent continuation of existing grants, supplemental requests, and new applications. Of those recommended for approval, 276 have been paid or designated for payment by the National Institute of Arthritis and Metabolic Diseases, in the amount of $5,508,432. These training grants are distributed among 84 institutions in 36 states, the District of Columbia, and Puerto Rico, and support approximately 389 indirect trainees.

Direct Traineeships

These awards for support during advanced training, are made by the Institute directly to physicians of demonstrated potential and a competence in an academic career, who are further qualified by at least three years of postgraduate training. They effectively complement the training grants program through provision of support in research method and related clinical and teaching skills. They are available in rheumatology, diabetes and metabolism, gastroenterology, hematology, physical biology, and related areas of research.

Requests		Approvals	
No.	Amount	No.	Amount
105	$ 567,291	87	$ 494,808

Of the direct traineeship applications recommended for approval in 1959, 77 have been paid or designated for payment to date, by the National Institute of Arthritis and Metabolic Diseases in the amount of $448,552. These direct traineeships, although made to individuals, geographically represent 40 different institutions in 17 states, the District of Columbia, Puerto Rico, and England.

Research Fellowships

The research fellowship program is an important component of the total training program. Postdoctoral and Special Fellowships provide individual support for research training in the basic and clinical sciences to persons upon whom degrees of Doctor of Philosophy and/or Doctor of Medicine have been conferred. It is complementary to the traineeship program, providing additional research training to meet the needs of individuals whose research interests are basic science oriented in the several specialized areas as above listed under Direct Traineeships. It is a mechanism whereby the biologist, chemist, or physicist is attracted to research endeavors essential to medical science.

In addition to the above mentioned fellowship programs, a very modest allocation of funds has supported fellowships of the Predoctorate type as a feeder to those categorical in nature. Emphasis has been placed upon the Post and Special Research Fellowship Programs as a means of most effectively carrying out the categorical aims of this Institute, utilizing the limited funds available to it in the fellowship program.

	Requests		Approvals	
Type	No.	Amount[a/]	No.	Amount[a/]
Predoctoral[a/]	67	$ 201,670	11	$ 33,115
Postdoctoral[a/]	130	792,090	33	201,063
Special[a/]	42	352,548	25	209,849

Averages

Predoctoral	$3,010
Postdoctoral	6,093
Special	8,394

a/ These are estimates based upon the average award for each type of fellowship.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

CALENDAR YEAR 1959

Summary Sheet

INTERDEPARTMENTAL COMMITTEE ON

NUTRITION FOR NATIONAL DEFENSE

INTERDEPARTMENTAL COMMITTEE ON NUTRITION FOR NATIONAL DEFENSE

Annual Project Report

Calendar Year 1959

This Committee was formed in 1954 as a result of presentation of a plan to establish the Committee by the Assistant Secretary of Defense (Health and Medical) and correspondence from the Operations Coordinating Board (OCB) staff which affirmed the desirability of forming such a committee and the usefulness of inter-agency coordination of various projects and studies on nutrition to avoid duplication of efforts among U.S. agencies. A Memorandum of Agreement was signed in 1955 by the Secretaries of the Departments of Defense, State, Agriculture, and Health, Education and Welfare, and the Director of the International Cooperation Administration. Subsequently, the Atomic Energy Commission became associated with the Committee. The program of the Committee was reviewed and again approved by the OCB in 1959.

The nutrition program directed by the Interdepartmental Committee on Nutrition for National Defense (ICNND), initiated in FY 1955 as a part of the U.S. Mutual Assistance Program, has contributed to our mutual security by the following means: (1) It has provided technical assistance in improving nutrition, food and health in the Armed Forces which has had a beneficial carry-over to the civilian populations. (2) It has increased efficiency of mobility of these Armed Forces due to improved utilization of their own food resources and development of emergency-type rations. (3) It has assisted in defining the major nutrition and feeding problems in the various countries. (4) It has assisted the countries concerned in establishing nutrition services by training local personnel and supplying the nucleus for a nutrition laboratory. (5) It has bettered U.S. friendship through medical, scientific and technical channels. All of these have supplemented the over-all U.S. foreign assistance program.

The proposed projects for FY 1961, estimated cost $350,000 include: (1) Completion of nutrition survey in Colombia, initiated May 15, 1960 ($30,500). (2) Nutrition surveys in Thailand and Lebanon. Official invitations requesting assistance in conducting nutrition surveys have been received through the State Department. Resurvey of the Armed Forces of the Republic of China (Taiwan), requested by the Chinese Government and U.S. MAAG, to evaluate the effectiveness of the rice enrichment program. This program was initiated as a result of the high prevalence of malnutrition noted in the Armed Forces during the survey conducted by the U.S. Army in 1954. (Surveys, Thailand, Lebanon and Taiwan: $202,500). (3) Since field activities of the surveys in Chile and Vietnam will not be completed until the latter part of FY 1960, $17,000 will be required in FY 1961 to complete the processing of data, analyses of food samples and preparation and presentation of reports. (4) Follow-up assistance to

Korea, [illegible] ceived [illegible] ($[illegible]). (5) [illegible] East-Africa regional program, which will cover [illegible] clinician consultant advice in Libya, Ethiopia, Iran, [illegible] and Spain ($46,000). (6) Meeting of the Fourth Armed [illegible] Nutrition Conference in the United States ($20,000). [illegible] held in Iran, 1956; Turkey, 1958; Pakistan, 1959). (7) South [illegible] Armed Forces Nutrition Conference, including Colombia, Ecuador, [illegible] Chile ($10,000).

To implement the nutrition program, upon receiving a formal [illegible] through State Department channels from an eligible country for [illegible] in conducting a nutrition survey, the request is coordinated with the Departments of State and Defense and the International Cooperation Administration before it is submitted to the Committee for approval. [illegible] approved the ICNND organizes a nutrition survey team, consisting of outstanding specialists in the fields of medicine, nutrition, [illegible] food technology and agriculture to conduct the survey. The duration of each survey is approximately 90 days. Data are collected, brought back to the United States, analyzed and discussed by the Committee and a final report with practical recommendations for improvement is sent to the country.

The follow-up program provides technical consultation to assist participating country in the implementation of the recommendations and related problems.

This is a cooperative, reciprocal program. The participating country furnishes personnel equal to or twice the number of the U.S. [illegible] for training in survey techniques. It also furnishes logistical support such as laboratory housing and transportation. To date, 25 United States universities and colleges, and the U.S. Army, Navy and Public Health Service have furnished over 100 doctors and specialists for the survey team. The program affords an excellent opportunity to learn much about these countries regarding nutritional disease, indigenous foods, food customs and practices. Such information is useful to our Armed Forces, U.S. Operations Missions and the Foreign Agricultural Service [illegible] current programs and in the event of an emergency.

Since January 1956 nutrition surveys have been completed [illegible] Pakistan, Korea, The Philippines, Turkey, Libya, Spain, Ethiopia, [illegible] Ecuador, Vietnam, and Alaska.

Institutes of Nutrition have been established in the Armed [illegible] of Iran, Pakistan, Turkey, Spain and Peru; and governmental institutes [illegible] nutrition in Ecuador, The Philippines and Ethiopia are actively [illegible] with the Armed Forces. At the Third Armed Forces International Nutrition Conference held in Pakistan, representatives from Iran, Pakistan and [illegible] reviewed the great progress these countries have made in ration [illegible] and development of rations for use in mobile situations. Much of the progress can be viewed as spectacular. A few illustrative examples point out some of the accomplishments: (1) Iran: They have conducted [illegible] nutrition surveys to evaluate the effectiveness of their ration [illegible] It has been possible to lengthen the training day for troops from [illegible] four-hour limit imposed by inadequate nutrition to a full eight-hour

A canning plant which has been reopened and is supervised by the Veterinary Corps of the Iranian Army has given an economic boost to the farmers in the area; it has resulted in the development of the first field ration, plus supplemental canned foods which have vastly improved troop mobility. A poultry industry has been initiated by the Director of the Nutrition Laboratory, with production of 20,000 poults per year. This has stimulated similar investments and has been used to supplement the feeding of the troops. (2) Turkey: New laws have been passed to provide for improvement in nutritional allowances and composition of the rations for the Turkish Armed Forces. Schools for training cooks and bakers and sanitarians have been established for their Armed Forces. Further surveys have been conducted to evaluate the effectiveness of ration changes. (3) Ethiopia: The personnel trained during the initial survey have been requested to study more extensively the nutritional status of the Armed Forces, and the Committee has been requested to assist in planning a ration for the Ethiopian Armed Forces. (4) Korea and Taiwan: Tremendous progress has been made in reducing the incidence of nutritional deficiencies by better utilization of their own resources; in addition, excellent progress has been made in developing field rations. (5) The Philippines: The Philippine Armed Forces for the first time have established a food and nutrition council to assist the Armed Forces in better ration planning and feeding of their troops. (6) General: The standard "Manual for Nutrition Surveys" published by the Committee in 1957, has been translated into Spanish (by the Spanish Armed Forces) and into French. This has been exceptionally well received by the Armed Forces and also civilian groups in this and in other countries as a standard reference book. The surveys have indicated the absence of any significant amounts of radioactive substances in urine samples of people or of food samples in the countries visited. The ICNND participated, upon request, in the United Arab Republic Food Conference in Cairo, Egypt, in November 1958 and in the Mid-East Annual Medical Symposium in Beirut, Lebanon, in April 1959. The Committee was instrumental in bringing together interested groups of American and Turkish students at the University of Illinois and American and Ethiopian students at the University of Wisconsin. (Members of the faculties of the Universities of Illinois and Wisconsin were on the Turkey and Ethiopia survey teams). The Secretariat has on record innumerable letters of appreciation from representatives of the host countries.