THE EFFECTS OF GLYCOCYAMINE-BETAINE ON THE PHOSPHATE FRACTIONS IN THE MUSCLES OF ADRENALECTOMIZED RATS

by

Rose Mary Boeck

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[redacted]

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

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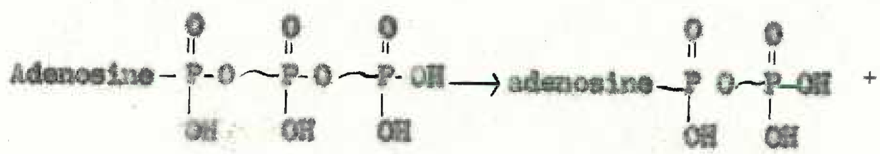
PREFACE

The demonstration in humans that certain clinical symptoms attributed to depleted body energy can be correlated with a decrease in the biochemical components of energy metabolism has led to investigations of effective methods of treating the deficiency (1). Glycocyamine plus betaine has been found useful as an adjuvant in treating the clinical syndrome. However, experimental proof of this effect rests with the development of laboratory test animals, low in energy reserves, and the demonstration that the therapy given will cause a significant change in these reserves. It has been shown in our laboratory that glycocyamine plus betaine will increase one of the energy components, creatine phosphate, in the muscle of rats as compared with controls (2). It appeared probable that the adrenalectomized rat might show depleted energy reserves because of the known effects of cortical hormone deficiency. Also, this animal might prove a suitable test animal to determine whether or not the effects of glycocyamine and betaine are mediated through the adrenal glands. A study on the biochemical mechanisms involved should consider at least three aspects of this problem. 1) Energy metabolism relations; 2) Possible effects of administered compounds; and 3) Standardization of test animals. These are the problems to be considered in this study.

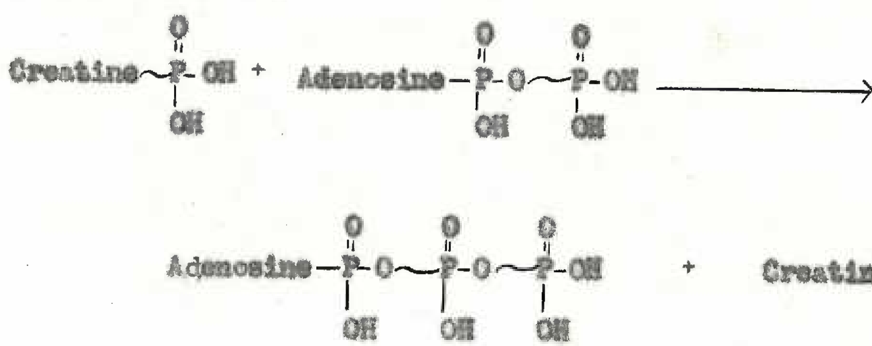
INTRODUCTION

I. Energy Metabolism Relations

Energy production, conservation and utilization are accomplished in the living organism by a group of unique organic molecules. These compounds possess chemical bond energy of the order of 10 - 14 kilocalories, which is readily transferable through enzyme systems for utilization in certain energy requiring processes. In muscle, the energy contained in the high energy phosphate bond of adenosine triphosphate (ATP) is converted into mechanical work through the contraction of the fibrils. Indirectly involved are the systems of energy production, largely through the tricarboxylic acid cycle, anaerobic glycolysis, and β-oxidation of fatty acids, and energy storage as creatine phosphate. The reaction directly yielding energy during muscle contraction may be indicated as follows:

1) 

Ortho phosphate
+
approximately 10 K energy

2) 

The ATP formed in reaction 2 can be utilized in reaction 1. Creatine phosphate serves as a reservoir of phosphate bond energy convertible into the phosphate bond energy of ATP. The overall effect in prolonged muscular activity is a depletion of creatine phosphate reserves. Restoration of these reserves occurs during the resting phase of muscle according to reaction 3:

$$3)\quad ADP + \sim PO_4 \longrightarrow ATP \rightleftharpoons Creatine \sim PO_4 \;+\; ADP$$

By various mechanisms during the processes of oxidation and glycolysis inorganic phosphate is converted to the high energy phosphate of ATP and creatine phosphate. Thus we see that the ability of a muscle to perform work is partially dependent on creatine phosphate which forms a reservoir of available energy for the rephosphorylation of adenosine diphosphate (ADP) to ATP.

The beginnings of our present concept of energy rich compounds may be traced to work reported in two publications in 1927. Eggleton and Eggleton(3) first noted a gradual increase in the optical density in their colorimetric procedure for the determination of muscle inorganic phosphate. This phenomemon was related to the existence of some organic phosphate which slowly degenerated to yield inorganic phosphate. They termed this labile compound phosphagen. Fatigued muscle contained less phosphagen than rested muscle and muscle in rigor contained little or none. Fiske and Subbarow(4), who had previously made similar observations, showed that the compound phosphagen is creatine phosphate, determined its empirical formula, and suggested the structure as it is known today. Although the work of Eggleton and Eggleton, and Fiske and Subbarow suggested that creatine phosphate is involved in muscular contraction, the finding of Meyerhof and Suranyi[5] that the energy content of creatine phosphate is of a higher order than the ordinary phosphate ester bond suggested a direct relation of this high energy compound to the operational energy in muscle contraction. This implied relationship was overlooked by Meyerhof and other workers during the late 1920's because of the well established theory that muscle contraction is related directly to the breakdown of glycogen and the formation of lactic acid. Lundsgaard[6] in 1930 proved this concept erroneous by showing that when glycolysis is blocked by iodoacetate, muscle contraction continues anaerobically until the creatine phosphate present is completely dephosphorylated. Lundsgaard[7] also showed that the energy from glycolysis is converted into phosphate bond energy.

Lohmann[8], in 1931, isolated adenosine triphosphate and later demonstrated that creatine phosphate serves to keep up the supply of ATP[9]. This process is represented by the equation:

$$\text{creatine phosphate} + \text{ADP} \rightleftharpoons \text{ATP} + \text{creatine}$$

This established that creatine phosphate is not the direct energy donor for muscle contraction, but serves as a store of energy rich phosphate groups for regeneration of ATP from ADP. However, this role of creatine phosphate is not merely a passive one. Goodall and A.G. Szent

Gyorgyi[10] have shown that creatine phosphate is an obligatory component of the muscle contraction and relaxation cycle. In their in vitro studies, they found that creatine phosphate is needed to produce complete relaxation of contracted muscle whereas the energy of ATP, even when present in excess, cannot be utilized for relaxation.

The energy carried by ATP largely originates from the oxidative mechanisms of the tricarboxylic acid cycle. As indicated in Figure I, the intermediate products of fat, carbohydrate and protein metabolism are introduced into the metabolic cycle by enzymatic conversion to cycle components, and ultimately oxidized to CO_2 and water. The energy yielded is conserved by transfer through a series of oxidative reactions that are coupled with phosphorylations to produce about 12 moles of ATP for every two carbon unit oxidized. Under anaerobic conditions, stored glycogen in muscle is degraded to hexose units (Figure II) which are further split to two phosphorylated triose molecules (Reaction 2). These are in equilibrium and the reaction continues through 3-phosphoglyceraldehyde to pyruvic acid with the production of 5 moles of ATP per triose (Reactions 3 and 4). Since one ATP is utilized in the phosphorylation of fructose-6-phosphate to fructose 1-6-diphosphate (Reaction 1), the overall gain in ATP is 9 moles per hexose degraded. In aerobic conditions, the dehydrogenation indicated in reaction 2 proceeds through the sequential reductions of the coenzyme diphosphopyridine nucleotide ($DPN + 2H \longrightarrow DPN \cdot 2H$) and flavoproteins, the latter finally being oxidized by electron transfer through the cytochrome system to form ATP and water as

FIGURE I

MUSCLE ENERGY PRODUCTION THROUGH TRICARBOXYLIC ACID CYCLE OXIDATIONS

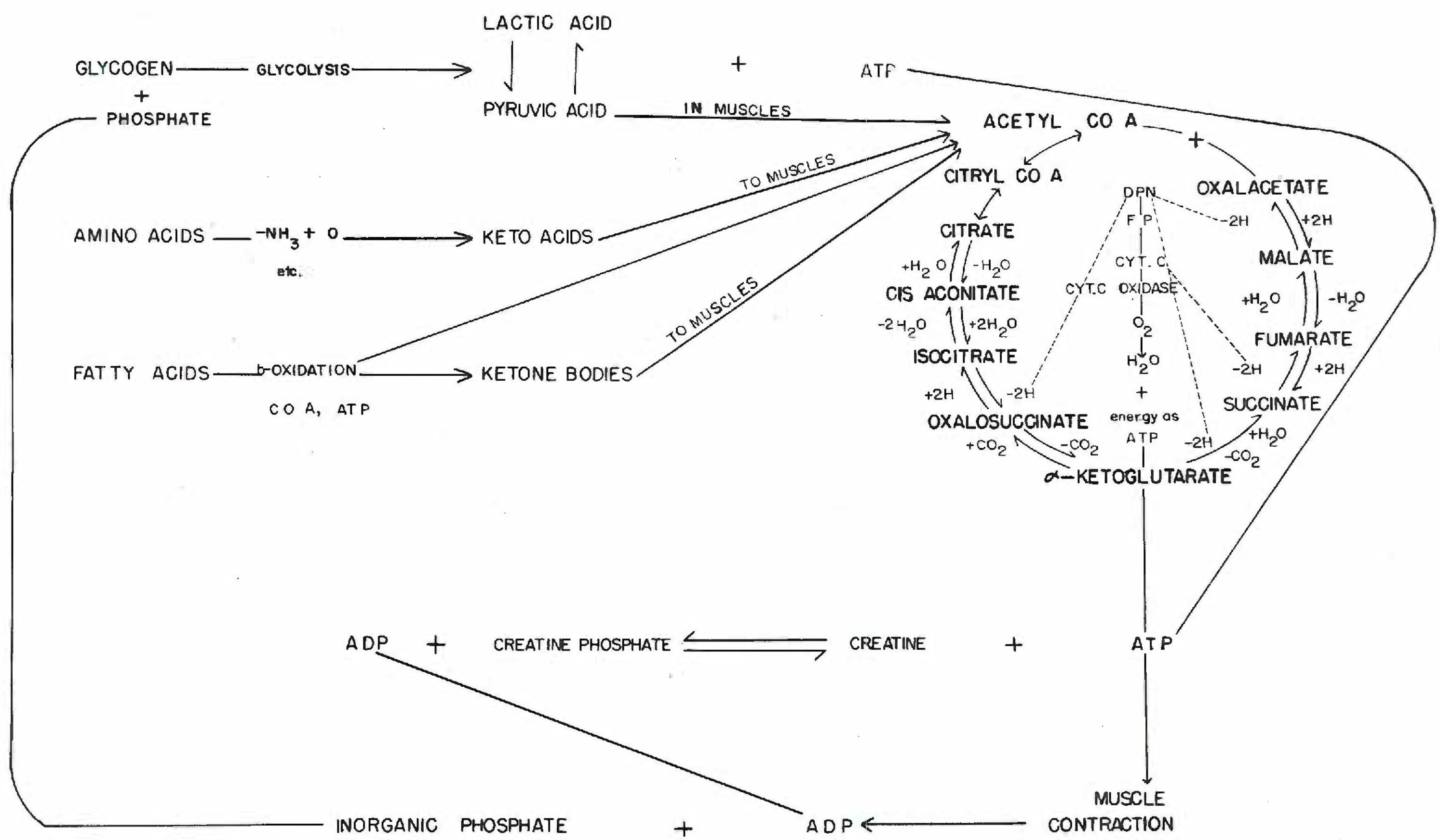
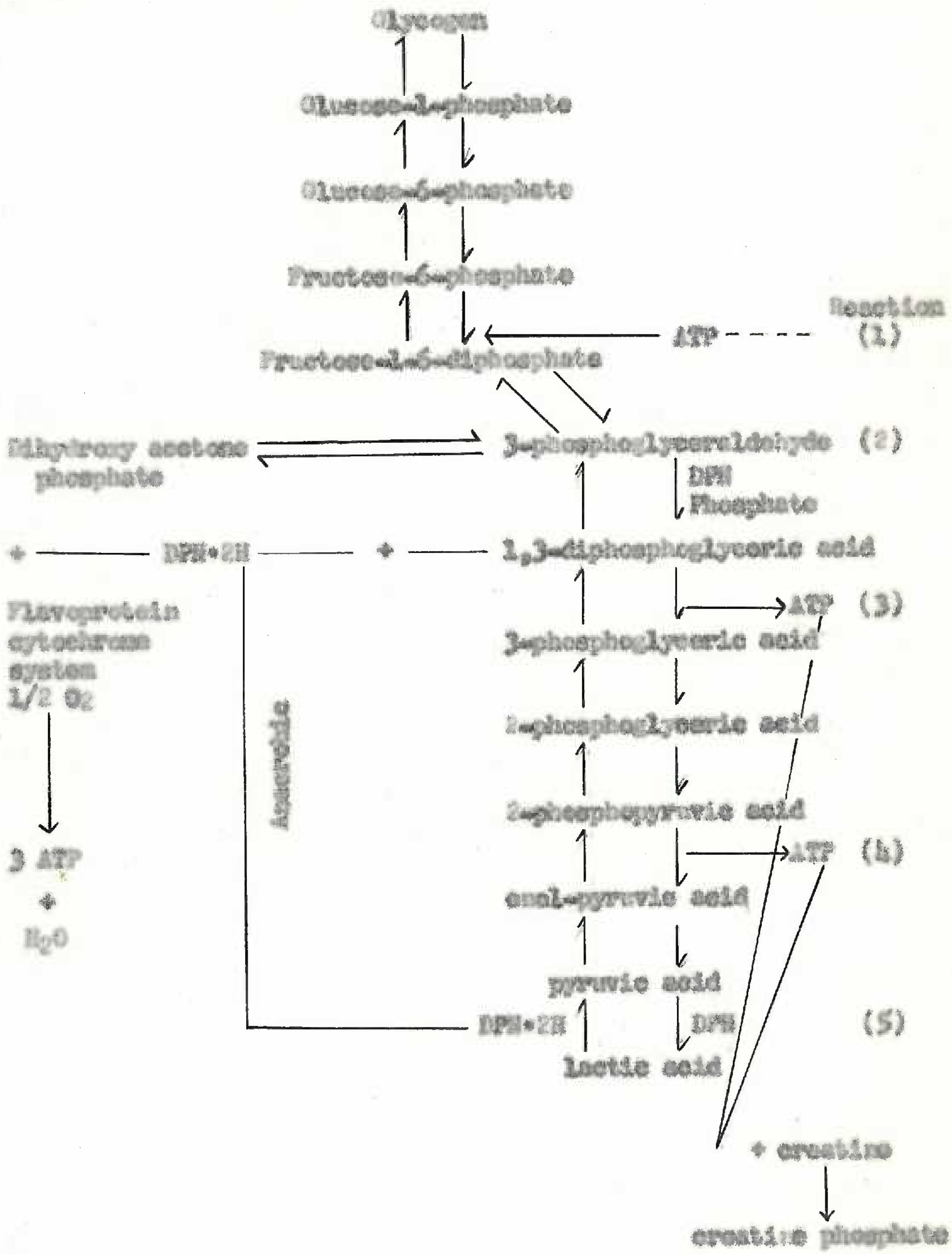


Figure II

Muscle Energy Production through Glycolytic Mechanisms



the overall end products. In anaerobic states, this oxidative system does not function and unless a comparable mechanism were available for oxidation, the glycolytic process would cease. However, anaerobic oxidation does occur through the transfer of H from DPN·2H to pyruvate (Reaction 5) with the formation of lactic acid. Thus it can be seen that the energy for a rapidly contracting voluntary muscle, which is under essentially anaerobic conditions, arises from two main sources, stored creatine phosphate and anaerobic glycolysis.

The type of work demanded of a muscle governs muscle structure and the mechanism of energy production. Slow, rhythmic contracting muscle needs a prolonged, steady supply of energy. The oxidative mechanisms largely involving oxygen answer this need because, under ordinary conditions, the oxygen supply to these tissues is adequate and until the available food products are depleted, energy production continues. Muscle responsible for short bursts of activity and requiring long periods of recovery is powered mainly by the quick energy yield from immediate breakdown of creatine phosphate supplemented by ATP from anaerobic glycolysis. Thus, as Szent Györgyi points out[11], the dark breast muscle of wild fowl is supplied with energy largely from oxidative mechanisms and therefore the bird is capable of prolonged flight. In contrast, the white breast muscle of domestic fowl depends to a greater extent on the creatine phosphate reserves and consequently, the chicken or turkey is capable of but short, violent barnyard flights.

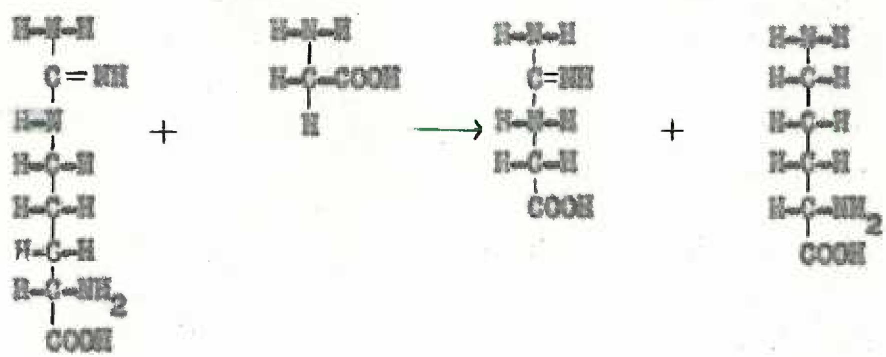
From these considerations, it can be seen that the relations of energy metabolism in muscle are indeed complex and a cursory review as presented here fails to indicate adequately the finer details of energy production and utilization. A point that should be reemphasized is the importance of creatine phosphate in muscle both indirectly as an energy donor to ATP, and directly as a necessary component to bring about muscle relaxation.

II. Possible Effects of Administered Glycocyamine-Betaine

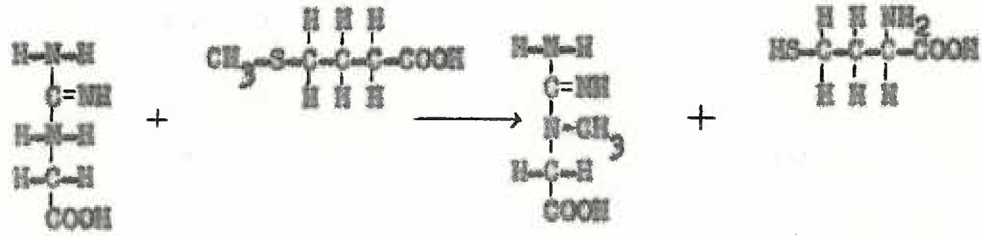
Glycocyamine plus betaine has been found to significantly raise the level of creatine phosphate in the muscle of rats(2). This action may be postulated to occur through an increase in free creatine formed through presentation to the organism of an excess of glycocyamine, or of the methylating agent (betaine), or by a combination of both effects. While methionine is the direct methylating agent in the formation of creatine, the methyl groups of betaine serve to keep up the supply of methionine by methylating homocysteine to methionine. The synthesis of creatine in vivo takes place in at least two main steps. The first is the formation of glycocyamine from arginine and glycine in the kidney, and the second is the methylation of glycocyamine by methionine to form creatine in the liver.

Several workers have shown the in vivo synthesis of glycocyamine and creatine by isotope tracer studies. Bloch and Schoenheimer(12) demonstrated that N^{15} glycocyamine is more active in labeling creatine that is N^{15} glycine. They also showed that the nitrogen content of creatine is from two precursors. When N^{15} ammonia was administered to rats, the isotope was present in the amidine moiety of creatine, but when N^{15} glycine was fed the methyl glycine fragment of creatine was labeled. du Vigneaud and his associates(13) showed that deuterium, when present in the methyl group of methionine fed to rats, could be recovered in the methyl groups of creatine.

This in vivo evidence of glycocyamine and creatine formation as obtained by Bloch and Schoenheimer and du Vigneaud was confirmed by the in vitro tissue slice studies of Borsook and Dubnoff. These workers showed(14) that liver slices from the cat, rabbit and rat can convert glycocyamine to creatine and that the presence of methionine enhances creatine formation. Borsook and Dubnoff further demonstrated (15) that the kidney is the exclusive site of glycocyamine synthesis from the amino acids arginine and glycine. Creatine synthesis may be shown to occur by the following equations:



Arginine + Glycine ⟶ Glycocyamine + Ornithine



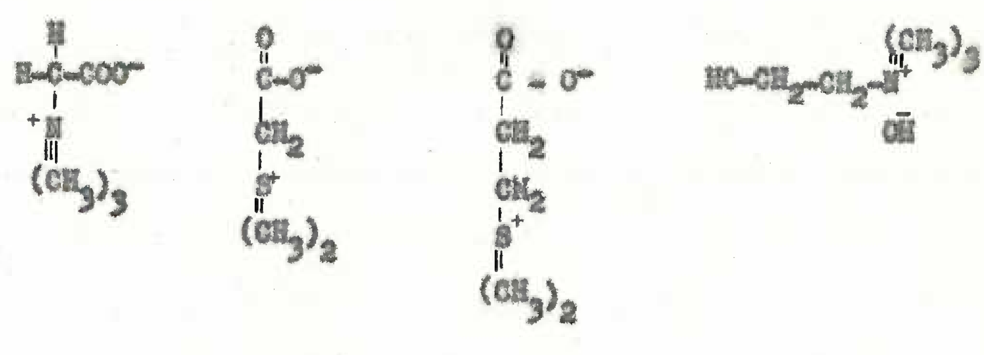
Glycocyamine + Methionine ⟶ Creatine + Homocysteine

Transmethylation from methionine to glycocyamine as well as to other methyl acceptors has been found to occur only in the presence of ATP in rat liver homogenates[16]. Cantoni[17] showed that ATP "activates" methionine to labilize its methyl radical, according to the reaction:

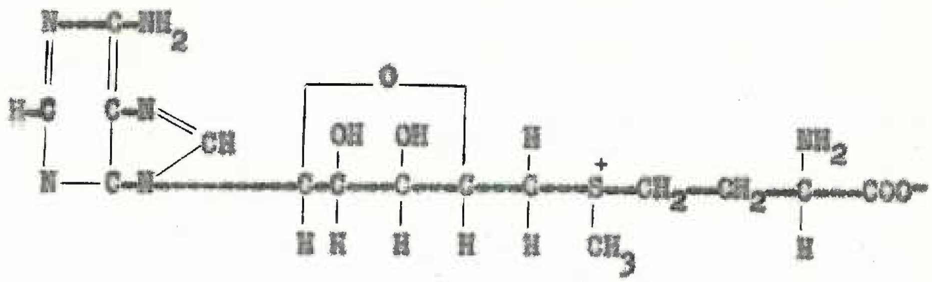
$$\text{l-methionine} + \text{ATP} \xrightarrow[\text{Mg}^{++}]{\text{GSH}} \text{"active" methionine} + \text{inorganic phosphate}$$

Glutathione and magnesium ions are necessary in the reaction. Cantoni also established that the "active" methionine is formed from the adenosine moiety of ATP with the liberation of ortho phosphate, and that "active" methionine can transfer its methyl group without the further action of ATP.

Betaine and its higher analogues, dimethyl thetin and propiothetin can effect methylation of homocysteine to methionine without activation by ATP[18]. In general, transmethylation to form methionine seems to be dependent on attachment of the methyl radical to an onium pole[19,20]. Betaine and its analogues and "active" methionine show this type of structure.



Betaine   Dimethylthetin   Dimethylpropiothetin   Choline



Active Methionine (S-Adenosyl methionine)

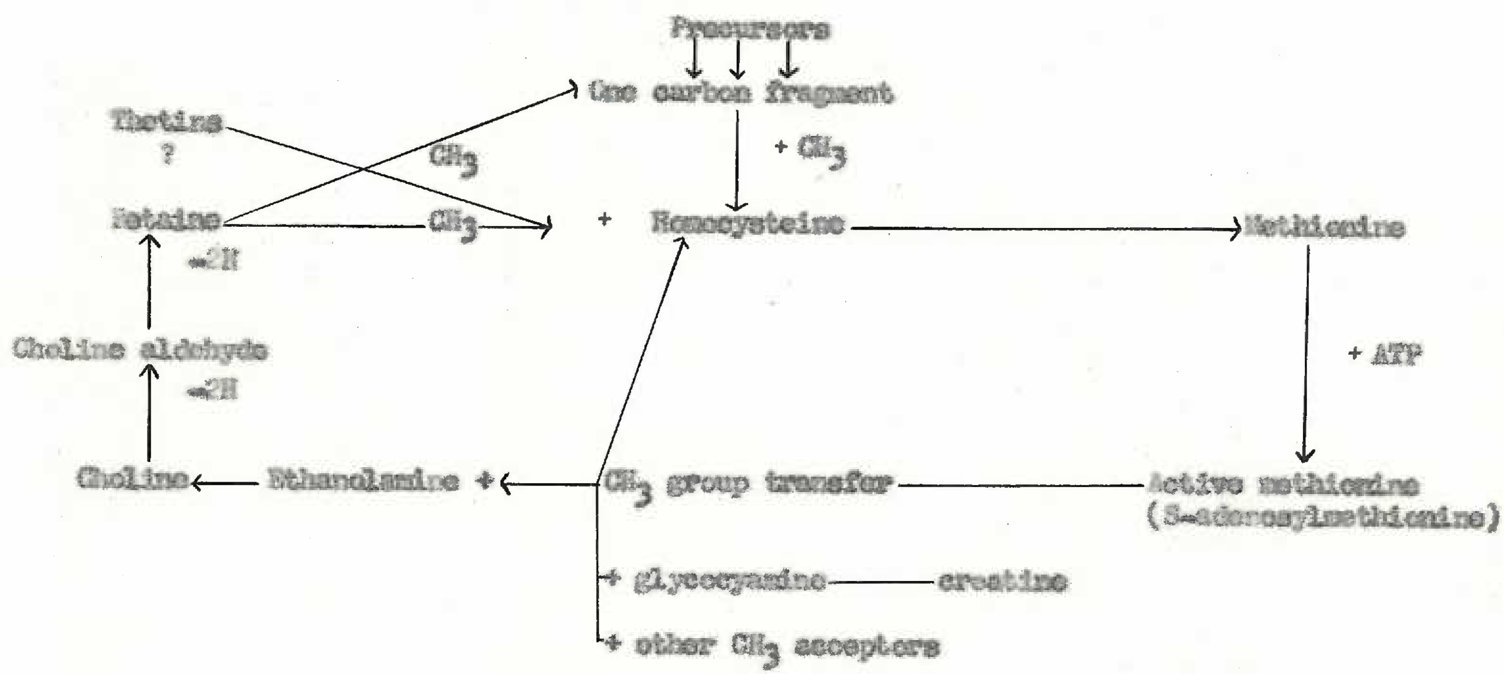
Choline does not have the onium pole structure, yet it has been found to be active in the biosynthesis of methionine. du Vigneaud et al.[21] investigated the ability of choline to replace methionine for rat growth and suggested that choline makes possible the in vivo methylation of homocysteine to methionine. This hypothesis was supported by Simmonds and associates[22] who fed rats choline labeled with deuterium in the CH_3 group and recovered the isotope in tissue methionine and creatine. The transmethylation appeared to be reversible as du Vigneaud et al.[13] had previously shown the methionine to choline methyl transfer. In the studies previously cited[21], du Vigneaud and associates found that betaine is able to substitute for choline in promoting rat growth in methionine deficiencies. In further studies[23], these workers synthesized deutero-methyl and N^{15} betaine and fed this isotope to rats. The labeled CH_3 appeared in tissue choline and creatine almost as rapidly as did the CH_3 from dietary deutero-choline. The N^{15} was partially recovered from tissue glycine and did not appear in choline in significant amounts. This indicated that betaine participates in methyl transfer without contributing appreciably to other choline components.

Proof that choline methylates through conversion to betaine was obtained by Muntz[24]. He incubated N^{15} choline with rat liver homogenates in the presence of homocysteine. If direct CH_3 transfer to homocysteine occurred, then isotopic dimethylaminoethanol from choline should have been recovered. However, the label appeared in the demethylation product of betaine, that is, in dimethyl glycine, and therefore indicated that labilization of choline methyl groups is effected through betaine. Dubnoff[25] has shown in rat liver homogenates that the oxidation of choline to betaine is mediated by the enzyme choline oxidase. Anaerobically, he could not demonstrate significant methylation of homocysteine by choline, but aerobically, the rate of transfer was similar to that for betaine. The theory of the obligatory onium pole attachment of CH_3 for methylation of homocysteine to methionine is valid for the methylation of homocysteine by choline because of the formation of the intermediate, betaine, which is the actual methylating agent. The reactions involving biological methylations are shown diagramatically in Figure III.

It is of interest that livers from certain animal species, including man, rabbit, and guinea pig show no choline oxidase activity[26]. Also, folic acid and vitamin B_{12} have been shown to influence reactions relating to methylation[27]. Deficiencies in these vitamins depress methylation reactions, but the exact mechanisms involved are unknown.

Figure III

Schematic Representation of Methyl Group Transfer



From these considerations, the possible effects of administration of glycocyamine plus betaine in increasing the levels of muscle creatine phosphate may be the result of providing the synthesizing mechanisms with an excess of glycocyamine and an additional source of labile methyl groups for increased creatine formation. This expanded energy storage potential could result in a proportional increase in creatine phosphate.

Dissociation of the two effects, energy production and energy storage constitutes a major problem. Because of the tendency of intact animals to maintain conditions of "normalcy" under stress, drastic changes in energy metabolism are needed before appreciable deficiencies are noted in the ATP-creatine phosphate system. The position of hormones in regulating metabolic function suggests a method of attacking the problem. The adrenal hormones have been shown to influence glycogen deposition and mobilization, protein catabolism, glucose utilization and other reactions generally relating to energy production. It was thought that the severe changes effected by removal of the adrenals in the rat might be reflected in significant changes in the components involved in the production and utilization of muscle energy. Evidence supporting this view may be found in the work of Ingle[28] who found that muscles from adrenalectomized rats have a lower work output than do muscles from intact control animals, and that this effect is partially restored by the administration of cortisone. Also, Verzar[29], in a review on the effects of corticoids on carbohydrate metabolism concluded that one of the main defects caused by removal of the adrenals is depressed enzyme activity, especially the phosphorylating enzymes.

Several workers have determined creatine phosphate and related compounds in muscles of adrenalectomized rats. Conflicting results are found in the literature. Helve(30) found a lower creatine phosphate level in the muscles of adrenalectomized animals as compared to the controls. Conway and Hingerty(31,32) found increased amounts of creatine phosphate in the muscles of adrenalectomized rats. Albaum and associates(33) have reported no significant change in high energy compounds after adrenalectomy.

Poor technique in preparing tissues for analysis may partially explain the variations in creatine phosphate levels. However, another possible cause may be the use of animals not completely deficient in the cortical hormones.

III. Difficulties Inherent in the Use of Adrenalectomized Rats—Importance of Determining the Presence of Functioning Adrenal Tissue.

When chronic adrenalectomized rats or mice are selected as test animals, the presence of true accessory tissue or regenerated adrenal capsule fragments left after operation poses a serious problem. Removal of the adrenal may cause the accessory tissue to hypertrophy and elaborate sufficient cortical hormone to destroy the effects of adrenalectomy in relation to the particular experiment under-taken. Versar[34] has stated that in his rat colony, 30% of the chronic adrenalectomized rats can be maintained without saline to drink, indicating the presence of functioning adrenal tissue. Speirs[35] found that about 62% of his adrenalectomized mice had hypertrophying accessory tissue. Therefore, the assumption that a chronic adrenalectomized animal is entirely without the effects of circulating cortical hormones may be questioned because accessory tissue may be producing the hormones. Before undertaking a study with adrenalectomized rats, it seemed advisable to develop a simple procedure to indicate the level of circulating adrenal cortical hormones.

Methods available to detect the presence of cortical hormones in small laboratory animals present a mixture of advantages and disadvantages. Sodium chloride withdrawal from adrenalectomized rats with subsequent death of the animal is adequate proof of adrenal insufficiency. This fact has been utilized by many workers [34, 36, 37] as a test for complete adrenalectomy. However, this method is unsuitable when the adrenalectomized animals are sacrificed in terminal experiments. Direct measurement of urinary or blood steroids as carried out by chemical[38,39,40,41] or biological assay methods[42,43] have been utilized as tests of adrenal function. These methods often require volumes of material not obtainable from small laboratory animals and are time consuming or extremely detailed.

Decreases in the number of circulating eosinophils and lymphocytes have been accepted as specific for the presence of increased amounts of adrenal cortical hormones[44,45]. In rats and mice, the effects of ACTH[46,47], epinephrine[48,49] nor epinephrine[50] and various other types of stress agents[51,52,53,54] have been found to cause a fall in eosinophils and lymphocytes in the intact, but not in the adrenalectomized animal. Adrenal function tests based on eosinophil or lymphocyte responses to stress have found wide acceptance[55,35,56]. Although this method would appear to be most suitable, objections may be raised because factors other than adrenal cortical hormones have been shown to modify eosinophil and lymphocyte counts. Dumm and Ralli[47] have shown that eosinophil response to stress may be modified by pantothenate deficiencies. Diurnal variations or handling the animals have been found to alter eosinophil and lymphocyte counts[45,56]. Also, it will be shown in this thesis that eosinophil and lymphocyte responses to stress, determined at varying times in the chronic adrenalectomized rat, do not represent reliable proof for the presence of accessory adrenal tissue. There is need, then, for a simple and accurate test for the presence or absence of functional accessory adrenal tissue. A water tolerance test for rats has been developed which fulfills these requirements. This test is based on the inability of the adrenal insufficient animal to handle administered doses of water in the normal manner. This observation was made in water intoxication and water balance studies on adrenalectomized and hypophysectomized animals as reported by Silvette and Britton[57], Swingle, et al[58], Gaunt and associates[59,60,61] and Dumm and Ralli[62]. The Robinson, Kepler, Power[63] test for adrenal insufficiency in humans is based on this principle. It will be shown that the water tolerance test is a more sensitive test for adrenal accessory tissue in the chronic adrenalectomized rat than the eosinophil and lymphocyte responses to stress.

A study was made of the phosphate fractions and total creatine in the muscles of adrenalectomized rats with and without glycocyamine-betaine treatment. Each adrenalectomized animal included in this study was water tested two days prior to sacrifice for analysis. Only those animals found to be without a significant amount of circulating cortical hormones as defined by the water tolerance test were included in the data.

METHODS

I. Animal Experiments

Female Sprague Dawley (180 - 250 g.) and Long Evans rats (175-460 g.) maintained on a Purina Chow diet were housed in individual metabolism cages in a constant temperature room at 24 - 26^{0} C. Sprague Dawley rats (200 - 250 g.) were used exclusively for determinations of the phosphate fractions in muscle.

A. Preparation of Adrenalectomized Animals

Adrenalectomized animals were prepared in a one stage operation by a retroperitoneal approach. 2.5 mg. per 100 g. body weight Nembutal was given by intraperitoneal injection. The Nembutal was diluted to contain 6 mg. per ml. in order to increase the accuracy in administering the small doses of anaesthetic required. After 10 minutes, an incision, 6 - 8 cm. long was made in the dorsal skin surface and the skin pulled back exposing the back muscles. A second smaller incision was made into the superficial muscles of the back, about 1 - 1.5 cm. below the 13th rib and about the same distance laterally from the spinal column, and the deep muscles separated by blunt dissection. The adrenal gland could usually be found on the upper pole of the kidney imbeded in the perirenal fat. The adhering tissue of the adrenal was grasped with forceps and brought outside the incision and separated by blunt dissection. All possible care was taken to prevent rupture of the adrenal capsule. A one stitch deep suture was taken to close the muscle incision. The second adrenal was removed in the same manner, except that a slightly lower muscle incision was made. The skin was then sutured and the rat given approximately 30,000 units of penicillin, intramuscularly. Rats so treated did not show symptoms of infection and the incisions healed readily. 24 hours after adrenalectomy, the animals appeared to have recovered fully from the effects of the operation. The rats were given either 0.5%, 0.7%, 1% or 2% sodium chloride in their drinking water.

B. Water Tolerance Test

Maintainence of uniform feeding conditions for the rats is essential to obtain consistent results, and ready access to food and water or saline until the period of fasting was important. Animals to be given the water tolerance test were fasted overnight (food removed around 5 p.m.; drinking water or saline not removed). The following morning, the rats were weighed and injected IP with warm water (5% by body weight). They were then placed in individual metabolism cages (Figure IV), without food or water, and the urine collected over a 5 hour period. The percent of injected water returned in 5 hours was calculated according to the formula:

$$\frac{\text{amount of urine excreted in 5 hours}}{\text{amount of water injected}} \times 100 = \%\text{ water returned}$$

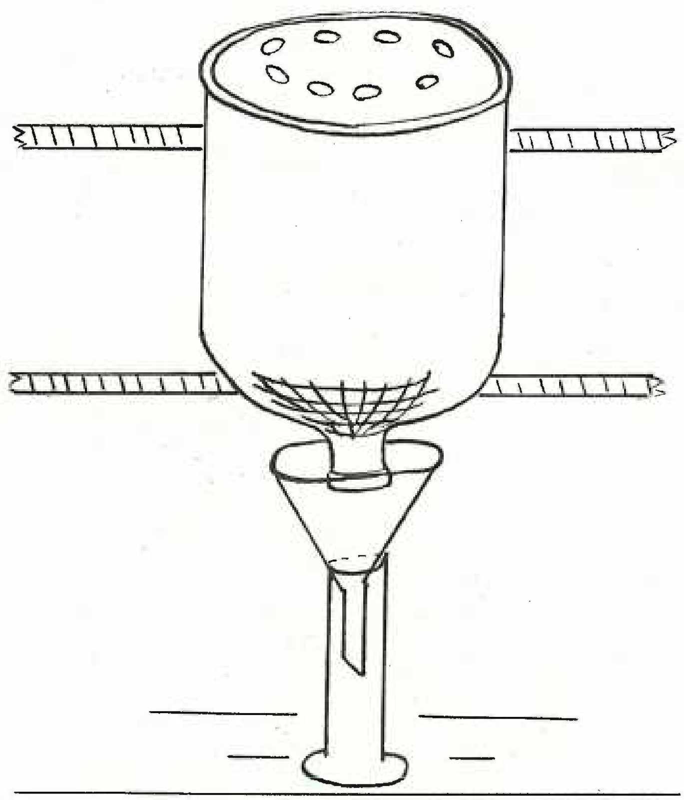
Water tests on 10 chronic adrenalectomized rats were run at 1 and 2 week intervals for 10 to 15 weeks. In addition, 20 animals were tested similarly for periods of 4 to 6 weeks, at which time they were sacrificed for determination of the phosphate fractions, total creatine and water content in the muscle.

C. Hematological Methods

Duplicate eosinophil, lymphocyte and total white blood cell

Figure IV

Metabolism Cage for Water Tolerance Test



determinations were made on blood obtained by cutting off the tip of the rat tail (discarding the first drop of blood) and bleeding directly into a porcelin plate well containing sodium oxalate crystals (2 drops of a 1.3% solution evaporated to dryness). The oxalated blood was drawn into the diluting pipettes immediately after bleeding the rat because evaporation and settling of the cells occurred within a few seconds causing errors in the cell counts. Standard white blood cell diluting pipettes and improved Brightline Spencer counting chambers were used for all cell counts. Care was taken to have all pipettes, counting chambers and cover slips free of dust or adhering particles and moisture.

Eosinophil Counts

Eosinophils per cubic millimeter were determined with Hinkleman's diluting fluid as given in the method of Fisher and Fisher (64).

0.5 gm yellow eosin
0.5 ml concentrated formalin
0.5 mg phenol
Distilled water to make 100 ml.

This diluting fluid was stored in the refrigerator and filtered immediately before use. The oxalated blood was drawn up to the 1 mark of a blood diluting pipette, the tip of the pipette carefully wiped free of excess blood, and Hinkleman's diluting fluid drawn up to the 11 mark. The pipette was rotated vigorously to mix the contents and prevent clumping of the cells. Minimum staining time for the eosinophils is from 3 to 5 minutes, however, as much as 3 to 4 hours may elapse without eosinophil degeneration. Prior to plating the diluted blood on the counting chambers, the pipettes were agitated for 2 to 3 minutes on a mechanical shaker to insure uniform distribution of the cells in the diluting fluid. After thorough mixing, the pipettes were removed from the shaker, about one third of the contents of the pipette discarded, the tip wiped free of excess fluid and the counting chamber carefully filled. The suspension was replated if the fluid ran over into the troughs of the chamber. After a few minutes to allow for the settling of the cells, the cells in the total ruled area of 9 sq. mm. were counted. The large granules of the eosinophils were deeply stained and refractile and could be easily seen with the low power lens. However, some cells of the polymorphonuclear neutrophil series contained granules that absorbed the stain and resembled eosinophils. Differentiation of the two types of cells was made with the high power lens by the sparse occurrence and smaller size of the granules of the segmented neutrophils. Also, certain artifacts were encountered that required identification with the higher magnification. Red blood cells were lysed by the hypertonic diluting fluid.

The number of eosinophils per cubic millimeter was found by multiplying the number of cells found in the 9 sq. mm. area by the factor 11.1. This factor is derived from the following formula:

$$\text{Cells/cu.mm.} = \text{No. of cells counted in 9 sq. mm.} \times \frac{1}{\text{area counted} \times \text{depth of counting chamber}} \times \text{dilution}$$

Depth of the counting chamber is 0.1 mm and the dilution is 10.

Lymphocyte Counts

Circulating lymphocytes were determined by two methods. One was the standard indirect method of determining the total number of white blood cells per cu. mm. and then calculating the absolute number of lymphocytes from a stained smear differential count. The second method was a new technique by which lymphocytes are stained and counted directly from a wet mount preparation. A modification of Greenthal's stain[65] was made and used as the diluting fluid.

0.01 gm crystal violet
0.10 gm methylene blue
3.0 ml glacial acetic acid.
Distilled water to make 100 ml.

This stock solution was filtered and diluted 1 to 10 with 5% acetic acid. Oxalated tail blood was drawn up to the 0.5 mark of a white cell pipette and the diluted Greenthal's stain drawn to the 11 mark. The procedure was then similar to the method of counting eosinophils with the exception that the cells in an area of 4 sq. mm. were counted. The nuclei of the white blood cells stain a deep blue and differentiation between the mononuclear and polynuclear cells is possible. The total number of stained cells and the number of mononuclear cells were determined. The total mononuclear (lymphocytes) and total white blood cells per cu. mm. are determined by the calculation:

$$\text{Cells/cu.mm.} = \text{no. of cells counted in 4 sq. mm.} \times \frac{1}{4 \times 0.1} \times 20$$

Because the wet mount method of counting circulating lymphocytes is a new technique, comparison was made between this method and the standard indirect method. Total white blood cells were determined with 5% acetic acid as the diluent and the counting technique was similar to that of the wet mount method for lymphocytes. Blood smears were treated with Wright's stain and a differential count made to determine the percent lymphocytes. Values for the two methods are given in Table I.

Table I

Comparison of Direct and Indirect Methods for Counting Total White Blood Cells and Lymphocytes in Sprague Dawley Rats

	No. of Counts	Average Absolute Counts: Direct	Average Absolute Counts: Indirect	Average Difference Between Direct and Indirect Methods	t^2	p^2
White Blood Cells	22	20,900	21,700	714±2290[1,2]	1.46	>.10
Lymphocytes	14	15,300	15,100	228± 1890	.45	>.10

1 Standard Deviation of the Difference

2 Calculated on the Basis of Paired Observations

There was no statistical difference between the counts as determined by the direct and indirect methods. The direct method was adopted for lymphocyte counts because the one step procedure was considered more satisfactory than the indirect two step method of white cell count and differential determination.

D. Ether Stress Test

Ether stress was administered to the rats as a method of inducing a fall in circulating eosinophils and lymphocytes. Blood counts were made on control or adrenalectomized rats between 9 and 10 a.m.; stress was administered and the counts repeated in 4 hours. The ether stress consisted of placing the rat in a covered glass jar containing a cotton pad saturated with ether until marked changes in the animal's respiration occurred. The rat was then quickly removed and allowed to recover until it became ambulatory. This stress procedure was immediately repeated twice in order to effect decreases in eosinophils and lymphocytes that would be reproducible.

E. Glycocyamine-Betaine Administration

200 mg. glycocyamine and 866 mg. betaine were mixed in 40 ml of water. Approximately 1 ml. of this suspension (containing about 4 mg. glycocyamine and 20 mg. betaine) per 100 g. body weight was administered by stomach tube 3 hours before the muscle samples were taken.

II. Preparation of Muscle Samples for Analysis

Rats were anesthetized by the intreperitoneal injection of a Nembutal solution containing 6 mg/ml in a dosage of 7.5 mg/100 g. body weight. Complete relaxation of the muscles usually occurred in 10 minutes. The animals were watched carefully for irregularity of breathing and signs of anoxia as indicated by blueness about the mouth and tongue. If anoxia was evident, the animal was discarded. Some rats were resistant to Nembutal and were given additional amounts of the anesthetic by intravenous or intraperitoneal injection. The skin above the lower leg joint was pulled away from the underlying tissue and dissected free with a minimum of trauma and bleeding. An incision in the skin was made to the groin along the inside of the leg. The rat was diapered with rubber sheeting to decrease fecal or urinary contamination and an anchor string was attached to the leg. The skin was pulled back and the leg placed immediately on a dry ice and acetone bed, and simultaneously the leg was pulled straight by the string and CO_2-acetone mixture poured over it (Plate I). The leg was packed in dry ice and acetone and allowed to freeze for 4 minutes (Plate II). Two operators are required in this procedure to insure maximum speed. After 4 minutes, the leg was cut off with chilled bone shears, wrapped in aluminum foil and stored in dry ice. Ordinary deep freeze temperatures will not prevent deterioration of the labile phosphate fractions. At temperatures below -40°C, however, it was demonstrated that tissues may be held for as long as two weeks without change in the phosphate fractions.

At the time of analysis, the lower leg joint was removed and the back part of the leg muscle separated from the bone with chilled bone shears. The outer layer of muscle was trimmed away and the muscle cut in half horizontally; duplicate samples were taken from the lower half. The upper portion was unsuitable for analysis because of a deep

Plate I

Position of the rat leg on the bed of dry ice and acetone immediately before additional dry ice and acetone mixture was poured over it from the beaker. Note the string attached to the leg which is pulled straight simultaneously as the leg is placed on the bed and freezing mixture poured.

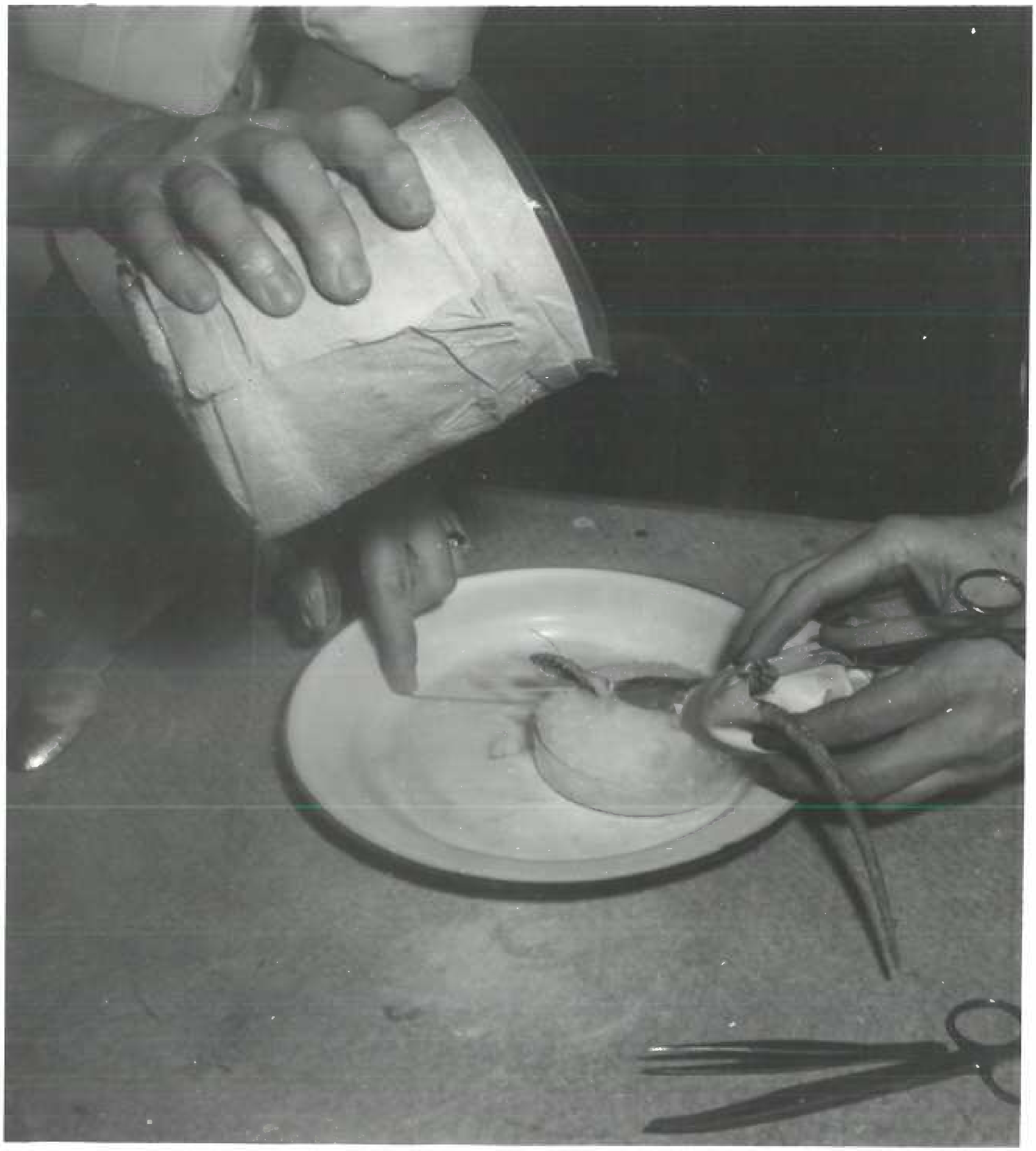


Plate IX

The rat leg, packed in dry ice and acetone and weighted with a beaker partially filled with the freezing mixture. Four minutes were allowed for thorough freezing of the leg.

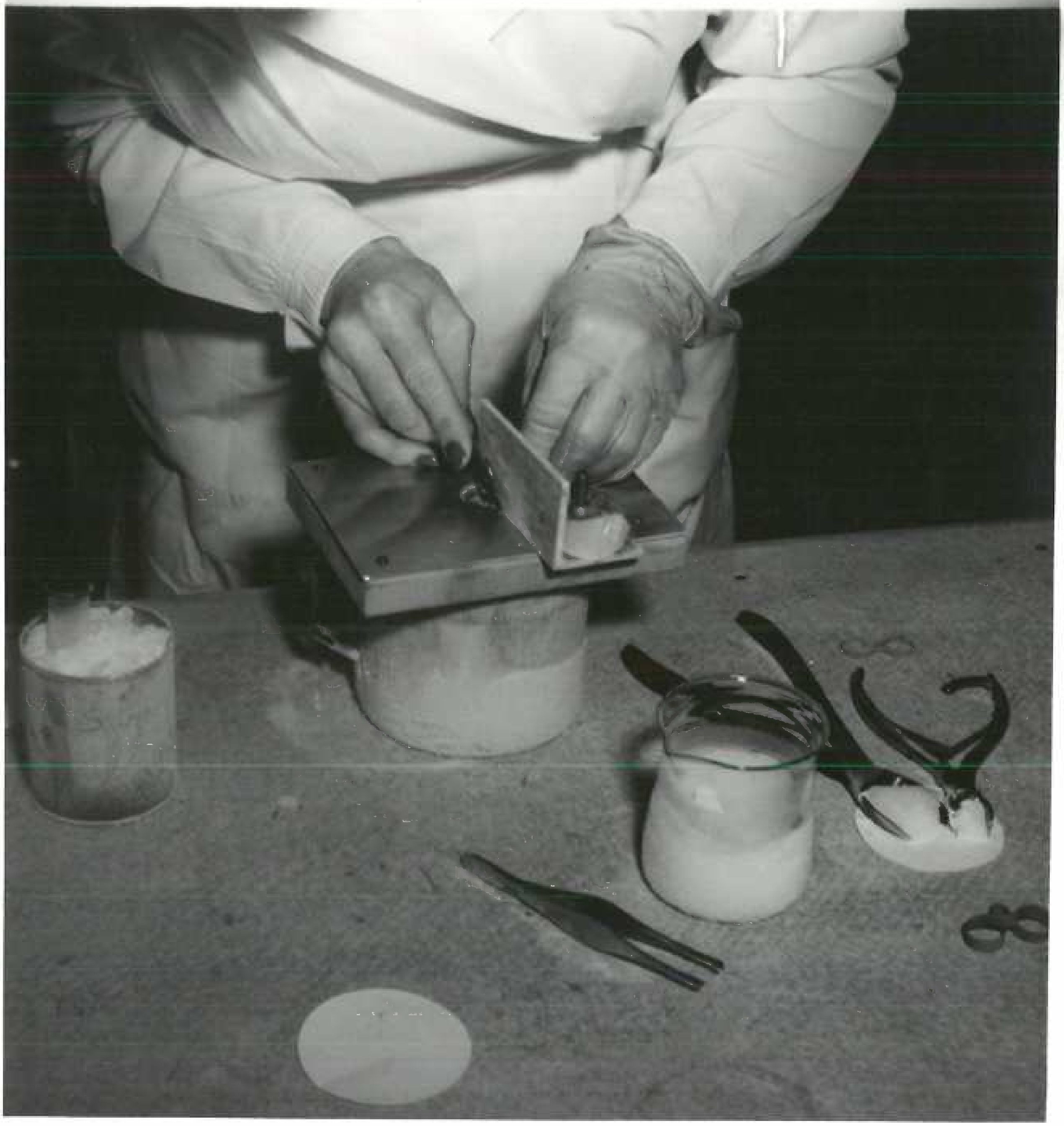
fat pad, indistinguishable from muscle in the frozen state. Samples from this area have been found to be diluted with fat. Contamination of the samples with bone fragments also causes inaccurate results. During the above procedure, the muscle was kept solidly frozen.

III. Preparation of Filtrate

Round bottomed 15 ml. centrifuge tubes, containing 2 ml. of 10% trichloroacetic acid (TCA), and fitted with rubber stoppers were packed in crushed dry ice for ten minutes. The tubes were wiped free of adhering moisture, quickly weighed and then repacked in dry ice. Tissues were sliced with an apparatus illustrated in Plate III. This consisted of a metal plate fitted with a movable piece of angled aluminum placed on a container partially filled with dry ice. The weighed tubes were inserted through the hole in the plate and rested on the dry ice. The muscle sample was held through an opening in the chilled angled aluminum and approximately 100 - 200 mg. samples sliced with a razor blade directly into the mouth of the tube because even brief contact with the air caused condensation of moisture on the thin tissue slices. Adhering pieces of tissue were wiped from the mouth and sides of the tube with filter paper, the stoppers replaced and the tubes repacked in dry ice for a few minutes. The tubes were quickly reweighed and replaced in dry ice. During the entire slicing procedure, all precautions were taken to keep the tissue sample crisply frozen. Boiling water baths and steam stills were not operated during the time when the tissues were sliced and weighed as moisture condensed on the frozen tissue and chilled tubes and caused serious weighing errors.

Plate III

Illustration of technique and equipment for slicing the frozen tissue. The tissue was held through an opening in the chilled aluminum angle, and sliced directly into the mouth of the weighed tube containing the frozen TCA. This tube had been previously inserted through the hole in the metal plate and rested on chopped dry ice. The weighed tube was kept in a container of dry ice (at the left) until it was placed in the slicing apparatus. Bone shears used to cut and trim the tissue were chilled with dry ice.



The weighed frozen tissue was then triturated with a footed glass rod after moistening the tissue with 3 drops of cold 10% TCA. When all the tissue had been partially broken up and appeared thoroughly wet, it was worked up the side of the tube with the rod and refrozen. With a second trituration, the frozen tissue was pulverized. The glass rod was rinsed with 8 ml. of 10% TCA (minus 3 drops), making a total volume of 10 ml. The rubber stoppers were replaced and the tissue homogenates were frozen and thawed three times with intermittent shaking to insure rupture of cell membranes. About 10 minutes were allowed for this process. The tubes were placed in an angle centrifuge and spun at 1800-2000 RPM for 2 or 3 minutes, the supernatant fluid filtered through Whatman #42 filter paper into tubes resting in dry ice. The filtrate was used for the determination of inorganic phosphate, creatine phosphate, ADP + ATP, and total creatine.

IV. Chemical Determinations

A. Inorganic Phosphate

Precipitation of inorganic phosphate was carried out according to the method of Fiske and Subarrow[4].

Reagents:

(1) Calcium chloride reagent. 10% Calcium chloride (13.3 gm. $CaCl_2 \cdot 2H_2O$ in 100 ml. distilled water) saturated with calcium hydroxide.

(2) 20% NaOH. 5 gm. NaOH in 25 ml. distilled water. Made fresh every two weeks and stored in a paraffin lined bottle to prevent silicate contamination from the glass.

(3) 10% Trichloroacetic acid

(4) Phenolphthalein indicator. 1 gm. phenolphthalein in 100 ml. of 95% alcohol.

Method: Three ml. aliquots of each sample of filtrate, pipetted immediately upon thawing of the frozen filtrate, were placed in 15 ml. graduated centrifuge tubes, and 20% NaOH added from a capillary dropper until the filtrate was alkaline to phenolphthalein (1 drop of the indicator to each tube). The solution was then back titrated with 10% TCA until a light pink color was obtained. A blank containing 3 ml. of 10% TCA was set up and treated in the same way as the filtrate. After neutralization, 1 ml. of 10% calcium chloride reagent was added and the sides of the tubes rubbed with glass rods to initiate precipitation of the true inorganic phosphate, the creatine phosphate remaining in solution. Because the calcium salt of ATP, formed under the conditions of this determination, begins to deteriorate after 10 minutes, liberating inorganic phosphate into the solution, the samples were allowed to stand exactly 10 minutes when the glass rods were rinsed down with distilled water and removed. The tubes were centrifuged at 3000 RPM for 4 minutes, carefully decanted and the precipitate washed with 4 ml. of distilled water and 1 ml. of the calcium chloride reagent. The samples were recentrifuged and drained a second time. The inorganic phosphate precipitate was dissolved in 2 ml. of 10% TCA, distilled water added to make 10 ml. and the phosphate content determined by the Gomori method to be described.

B. Creatine Phosphate

The difference between the true inorganic phosphate precipitated by calcium, and the phosphate found in the TCA filtrate was taken as creatine phosphate phosphorus. Two ml. aliquots of the filtrates and 2 ml. of 10% TCA for a blank were pipetted into 15 ml. graduated centrifuge tubes, made up to 10 ml. with distilled water, and the phosphorus content determined.

C. ADP + ATP

The adenosine diphosphate plus adenosine triphosphate fraction of the TCA filtrate was found by determining the phosphate present after a 7 minute hydrolysis (100°C in 1 N HCl) and then subtracting from this value, the amount of phosphate found in the TCA filtrates. One ml. aliquots of the samples and 1 ml. of 10% TCA for a blank were pipetted into 15 ml. graduated centrifuge tubes, 0.2 ml. concentrated hydrochloric acid added to each, and the volume made to 2.5 ml. with distilled water. The tubes were covered with marbles, placed in a boiling water bath for 7 minutes, and then cooled in running tap water. The volume was adjusted to 10 ml. with distilled water and the phosphate content determined.

D. Determination of Phosphate

Phosphate determination was according to the method of Gomori[66].

Reagents:

(1) Molybdic-sulfuric reagent. Mix 2 volumes of a 5% solution of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), 1 volume of 10 N sulfuric

acid and 1 volume of distilled water.

(2) Reducing solution. 1 g. Elon (p-methylaminophenol sulfate) in 100 ml. of a 3% sol tion of sodium bisulfite.

Method: 2.5 ml. of molybdic-sulfuric acid reagent and 1 ml. of reducing solution were added to each tube containing 10 ml. of solution from the above procedures. The volumes were adjusted to 15 ml. with distilled water, the tubes fitted with rubber stoppers and thoroughly mixed by inversion. Maximum optical density of the solution was reached in 45 minutes and was stable for a total of 90 minutes from the time of mixing. The solutions were transferred to 19 mm. cuvettes and read in the Coleman Junior Spectrophotometer at 690 mμ. Each set of samples was read with its respective blank set at 100% transmission. The mg. of phosphorus was determined from a calibration curve made with KH_2PO_4. Complete adherence to Beer's law was found in concentrations of 0.005 to 0.08 mg. of phosphorus.

Calculation: Mg. phosphorus in sample $\times \frac{100}{\text{tissue weight}} \times \frac{10}{\text{ml. of filtrate used}}$

$$= \text{mg. P/100 gm. tissue}$$

E. Creatine Determination

The 3,5-dinitrobenzoic acid method of Langley and Evans[67] for determining urine creatine was modified for determination of tissue creatine. The Langley and Evans method is based on the principle that creatinine, when treated with the sodium salt of 3,5-dinitrobenzoic acid in alkaline solution, produces a garnet red color (Bolliger reaction), which is proportional to the amount of creatinine present.

Creatine was converted to creatinine by heating with acid and the amount of chromogen present was taken as the creatine content. The creatinine content of the muscle was included in the total creatine values since it was found that the creatinine content is relatively very small.

The Langley and Evans method of determining creatine was selected in preference to the Jaffe picric acid method(68), because it was found that glycocyamine will react in the Jaffe method to produce color and interfere with the determination of creatine plus creatinine. Since glycocyamine was to be given as a test substance, it was considered essential that the method for determination of creatine in the muscle show no reaction with glycocyamine. When glycocyamine was added to standard creatinine solutions in concentrations 5 times higher than that of the standard solutions (from 0.01 to 0.09 mg. creatinine) and the determinations carried out by the methods of Jaffe and Langley and Evans, the 3-5-dinitrobenzoic acid reagent did not react significantly with the added glycocyamine, that is, the standard solutions with glycocyamine did not show additional chromogen present. With the Jaffe method, however, the addition of glycocyamine raised the reading of the standard solutions, demonstrating the non-specificity of the picric acid reagent for creatinine.

Reagents:

(1) 3,5-dinitrobenzoic acid reagent. 25 ml. of 10% aqueous solution of Na_2CO_3 (anhydrous) was added slowly with constant stirring to 10 g. 3,5-dinitrobenzoic acid, 75 ml. distilled

water was added, and the mixture heated just to boiling to dissolve the solids. A satisfactory solution had a pale yellow color with no tinge of red or brown and showed no increase in color when 0.8 ml. of the reagent, 2 ml. distilled water and 0.4 ml. 1 N NaOH were mixed. Recrystallization from 80% alcohol according to the method of Langley and Evans[67] may be necessary with impure dinitrobenzoic acid. Eastman Kodak Company 3,5-dinitrobenzoic acid has been found satisfactory.

(2) 3 N NaOH

(3) 1 N HCl

(4) 10% TCA

(5) Stock standard creatinine solution (10 mg. creatinine/1 ml. in 0.1 N HCl). Dilute working standards (0.01 and 0.04 mg/ml in 0.1 N HCl).

<u>Preparation of Calibration Curve</u>

Aliquots of standard solutions containing from 0.01 to 0.08 mg. creatinine were pipetted into test tubes calibrated at 15 ml., 1 ml. of 10% TCA and 0.4 ml. 1 N HCl were added and the volume adjusted to 3 ml. with distilled water. A blank was prepared containing 1 ml. 10% TCA and 0.4 ml. 1 N HCl and diluted to 3 ml. with water. The tubes were covered with aluminum foil, heated in a pressure cooker for 30 minutes at 15 to 20 lbs. pressure and then cooled in running tap water. Six drops 3 N NaOH were added from a dropper calibrated to deliver 35 - 40 drops per ml., and the volume adjusted to 10 ml. with distilled water. Three ml. of dinitrobenzoic acid reagent and 0.4 ml. 3 N NaOH were added, the final volume was made to 15 ml. and the tubes mixed thoroughly and the solutions were transferred to 19 mm. cuvettes. Because the color reaction is light sensitive, 7 to 10 minutes in the dark at room temperature were allowed for full color development. The solutions were read in the Coleman Junior Spectrophotometer at 495 mu, the cuvettes being kept in a covered box during the reading process. The color developed was stable for 3 - 5 minutes at the concentration of alkali described above. Too rapid color development occurred with higher concentrations of NaOH and with too little alkali the color development was slower and not proportional to the creatinine content. Recoveries of known amounts of creatine averaged 100% for 9 determinations (range 96 - 107%).

Determination of Creatine in Muscle Filtrates

One ml. of the TCA filtrate prepared for the phosphate determinations and 1 ml. 10% TCA for a blank were pipetted into test tubes graduated at 15 ml., 0.4 ml. 1 N HCl was added and the procedure described above for the calibration curve carried out. Because of the sensitivity of the color reaction with the dinitrobenzoic acid reagent, known solutions of creatinine were run with each tissue determination to check against the calibration curve.

Calculation:

$$\text{Mg. creatinine in sample} \times \frac{100}{\text{tissue weight in grams}} \times \frac{10}{\text{ml. of filtrate used}} \times 1.16 = \text{mg. creatine/100g. tissue}$$

The color intensities of the solutions were dependent upon the concentrations of the 3,5-dinitrobenzoic acid. A decrease in temperature causes a decrease in the amount held in solution and a consequent lower reading in standard solution values. This variation was controlled by gently warming the reagent to redissolve any precipitate.

F. Dry Weights

After the two tissue samples had been sliced and analyzed, the remaining muscle of the rat leg was thawed in the aluminum foil wrappings. Fat and fasciae were removed, and the muscle tissue cut in small pieces and dried to constant weight (24 hours at 90°C).

$$\frac{\text{Weight of Dry Tissue}}{\text{Weight of Wet Tissue}} \times 100 = \text{Percent Dry Weight}$$

RESULTS AND DISCUSSION

I. Water Tolerance Tests

Water tolerance tests administered to 33 control Sprague Dawley rats gave values (Table II) from 57 to 110% with a mean at 81% and a standard deviation of 13.0. From these control figures, the limits

Table II

Comparison of Water Tolerance Values in Control Sprague Dawley and Long Evans Rats

	N	Mean	t	P
Long Evans	15	66.7 ± 3.20*		
Sprague Dawley	33	81.0 ± 2.26	3.57	$<.005$

* Standard Error

of the positive water tolerance test were set as 42 to 120% (81 ± 3 times the standard deviation 13.0). A positive test for the presence of functioning adrenal cortical tissue is indicated by a return of 42% or more of injected water, and values of 41% or less water return are considered negative. There appears to be a significant species variation to the water tolerance test. Table II shows that 15 control Long Evans rats gave water tolerance test values averaging 67% (range 48 to 91%). The difference in water return values between the Sprague Dawley and Long Evans rats was found to be statistically significant $P < .005$. Because of this species variation and the possible influence of other factors, it is evident that the limits of the water tolerance test as obtained from experiments with rats in this study apply only to Sprague Dawley animals maintained under conditions similar to those described.

In order to establish the validity of the water tolerance test for the presence or absence of accessory adrenal tissue, a comparison was made of the results of the water test before and after adrenalectomy with accepted methods of detecting circulating cortical hormones. For reasons previously cited, the methods selected for the comparison were the eosinophil and lymphocyte responses to stress. A method of inducing stress in the rats was needed to cause significant and consistent decreases in the levels of circulating eosinophils and lymphocytes in intact animals. Various stress procedures were tried such as injection of hypertonic saline, intermittent electric shock, and tossing the animals in the air. These methods were rejected because they did not produce sufficient stress or because they were technically unsuitable.

ACTH administration has been shown to decrease the levels of circulating eosinophils in intact rats presumably by stimulation of the adrenals to produce increased secretion of cortical hormones. Intramuscular injections of ACTH (7 to 10 units) caused decreases in blood eosinophils in 9 of 10 animals. (Table III). The percent change 4 hours after ACTH administration ranged from +48 to a -50. Forsham[69] has suggested that ACTH given intramuscularly may be partially inactivated during the absorption process. In addition, certain preparations of ACTH have shown assay values below the stated unit strength[70]. Therefore, intracardial, and intravenous injections of ACTH, assayed by the Vitamin C method, were given to two series of rats. When ACTH was administered intracardially the percent change in eosinophils ranged from +25 to -76, and when given intravenously, from +85 to -83. (Table III).

Table III

Eosinophil Response 4 Hours After Injection of ACTH[1] (7-10 U) in Control Rats

Intramuscular[2]			Intracardial[2*]			Intravenous[3]		
Absolute Counts		%	Absolute Counts		%	Absolute Counts		%
Before	4 hr. after	Change	Before	4 hr. after	Change	Before	4 hr. after	Change
733	411	-44	211	222	+5	190[4]	171	-9
1290	777	-40	777	488	-52	288[5]	534	+85
356	266	-25	744	933	+25	522[5]	187	-79
533	378	-29	1988	366	-33	466	111	-76
800	1021	+28	1710	599	-66	622	222	-64
566	299	-47	511	278	-46	611	255	-58
1021	833	-18	466[3]	111	-76	300	144	-52
1022	555	-39	311[3]	266	-14	621	688	-14
1078	533	-50				322	100	-83
510	278	-46				300	244	-22
						211	167	-21
						233	155	-33
						233	111	-52

[1]Armours ACTHAR courtesy Dr. J. D. Fisher; ACTH assayed by Vitamin C method.
[2]Long Evans rats. [2*] Long Evans rats—light ether anesthesia. [3] Sprague Dawley rats—light ether anesthesia. [4] 2.5 Mg / 100 g i.p. Nembutal. [5] No anesthesia.

Ether stress produced decreases in circulating eosinophils that were comparable to those caused by ACTH administration (Table V, column 5). Because of the risk involved in the intracardial injections and the necessity of ruling out those animals in which the intravenous injections were not completely satisfactory, the ether stress method was adopted for subsequent tests.

In order to define the limits of the variation in eosinophil counts caused by indeterminate sources of error such as diurnal variation, clinical condition of the animals and technique of counting the cells, repeat determinations of the response to ether stress were made within 1 to 3 weeks. The eosinophil response to ether stress in the individual rats showed wide variability. Rats 2, 5, and 6 (Table IV) were apparently resistant to the standard stress conditions and showed a low grade response to stress. Rats 7, 8, and 9 exhibited a greater degree of eosinopenia. However, the individual rat showed a rather consistent response to stress, the difference between the two experiments being 2 - 23%.

Hungerford[71] and Elmadjian and Pincus[54] have regarded the lymphocyte response to stress a criterion of adrenal cortical activity. However, Table IV shows that there is greater variation in lymphocyte counts (0 - 41%) in the individual rat on repeat exposures to stress than in the eosinophil counts. Winters et al[72] in studying eosinophil and lymphocyte responses to epinephrine and ACTH in pantothenate deficiencies, concluded that the lymphocyte determinations were less reliable than the eosinophil counts, and suggested that possible defects in technique in counting the lymphocytes (indirect method) might be responsible for their apparent lack of sensitivity to stress. In the work reported in

Table IV

Eosinophil and Lymphocyte Responses to Repeat Exposures to Ether Stress in the Same Control Rat
(Within 1 - 3 Weeks)

Rat	Eosinophils Before Stress	Eosinophils After Stress	% Change After Stress	Difference Between 2 Experiments	Lymphocytes Before Stress	Lymphocytes After Stress	% Change After Stress	Difference Between 2 Experiments
1	278*	244	-12	13	9,400*	10,700	+14	0
	255	200	-25		9,800	11,200	+14	
2	244	244	0	3	19,500	21,400	+10	4
	377	366	-3		17,600	18,600	+ 6	
3	222	189	-15	11	6,700	4,900	-27	13
	378	278	-26		9,100	5,500	-40	
4	977	1190	+22	23	8,700	10,100	+16	39
	1021	1010	- 1		9,200	7,100	-23	
5	566	577	+ 2	5	15,600	13,600	-13	13
	866	806	- 3		17,300	12,800	-26	
6	322	344	+ 7	20	15,400	19,400	+26	25
	1890	1642	-13		12,400	12,500	+ 1	
7	300	156	-48	2	18,800	13,600	-28	41
	244	122	-50		16,000	18,500	+13	
8	322	288	-38	17	19,400	17,600	- 9	5
	244	110	-55		17,800	17,100	- 4	
9	200	133	-33	4	11,400	9,800	-15	3
	344	244	-29		13,500	11,400	-18	
Average difference				11				16

* Absolute counts, each figure is the average of duplicate samples of blood.

this thesis, even with a direct method of counting the lymphocytes, the same lack of sensitivity was encountered.

Rats used in the comparison study were selected on the basis of their eosinopenic response to ether stress. Only those animals were included in this study whose initial eosinopenia was 20% or greater (one rat with an initial response of 17% was included). This procedure insured that the changes in eosinophils and lymphocytes produced by the stress would be sufficiently high to show significance above the changes caused by technique, diurnal variation, etc. The screening of rats according to eosinopenic response to stress has been reported by Heming, Sax and Holtkamp[73] as a means of increasing the sensitivity of their assay procedures. Rosemberg et al[74] have reported a similar procedure in which adrenalectomized mice were selected for steroid bioassay on the basis of eosinophil response to ACTH.

The results of a comparison of water tolerance test values with circulating eosinophil and lymphocyte response to stress before and 1 to 3 weeks following bilateral adrenalectomy are given in Table V. This table shows a decrease in water return following adrenalectomy in every instance, 52 to 90% less water being returned. The average values for water returned were 78.7% before and 0.7% after adrenalectomy. The fall in eosinophils produced by stress in the intact animals was changed to an average increase in eosinophils of 74% post-adrenalectomy. Variation in the individual rats before and after adrenalectomy ranged from 46 to 207% (rats 3 and 10) with a mean at 114%. These changes are evidently of a greater magnitude than those found in repeat experiments on 9 rats (range 2 to 23%, mean 11) Table IV.

Table V

Comparison of the Water Tolerance Test and the Eosinophil and Lymphocyte Responses to Ether Stress Before and After Adrenalectomy (Sprague Dawley Rats)

	Control					Adrenalectomized				
		Absolute Counts		% Change After Stress			Absolute Counts		% Change After Stress	
Rat	% Water Returned	Eosin.	Lymph.	Eosin.	Lymph.	% Water Returned	Eosin.	Lymph.	Eosin.	Lymph.
1	99	300 (156)[1]	18,800 (13,600)	-48	-28	20	378 (440)	15,700 (17,200)	+18	+10
2	70	322 (200)	19,400 (17,600)	-38	-9	18	244 (650)	16,100 (25,400)	+164	+58
3	52*	322 (266)	23,100 (25,400)	-17	+10	0	222 (650)	16,600 (20,900)	+190	+26
4	76	266 (133)	19,300 (14,500)	-50	-24	12	200 (356)	13,100 (18,100)	+78	+38
5	85	333 (222)	16,300 (19,900)	-33	+22	0	255 (366)	16,800 (21,100)	+43	+25
6	90	266 (178)	11,500 (15,700)	-33	+37	0	623 (788)	14,800 (19,600)	+27	+32

*Not included in control figures because water not available during fasting period.
[1]Figures in parentheses are the absolute counts taken 4 hours after ether stress.

Table V (cont.)

Comparison of the Water Tolerance Test and the Eosinophil and Lymphocyte Responses to Ether Stress Before and After Adrenalectomy (Sprague Dawley Rats)

	Control					Adrenalectomized				
Rat	% Water Returned	Absolute Counts Eosin.	Absolute Counts Lymph.	% Change After Stress Eosin.	% Change After Stress Lymph.	% Water Returned	Absolute Counts Eosin.	Absolute Counts Lymph.	% Change After Stress Eosin.	% Change After Stress Lymph.
7	77	288 (133)[1]	17,900 (13,900)	-54	-22	0	244 (366)	8,800 (9,700)	+50	+10
8	82	272 (173)	13,800 (9,100)	-45	-35	9	477 (898)	16,500 (15,500)	+88	- 6
9	—	260 (199)	13,100 (10,100)	-45	-27	9	827 (1449)	14,400 (18,200)	+75	+26
10	77	173 (116)	12,700 (11,000)	-35	-14	0	422 (466)	13,600 (14,600)	+11	+ 7
Average	78.7	250 (118)	16,590 (15,080)	-40	- 9	0.7	389 (643)	14,640 (18,030)	+74	+23

[1]Figures in parentheses are the absolute counts taken 4 hours after ether stress.

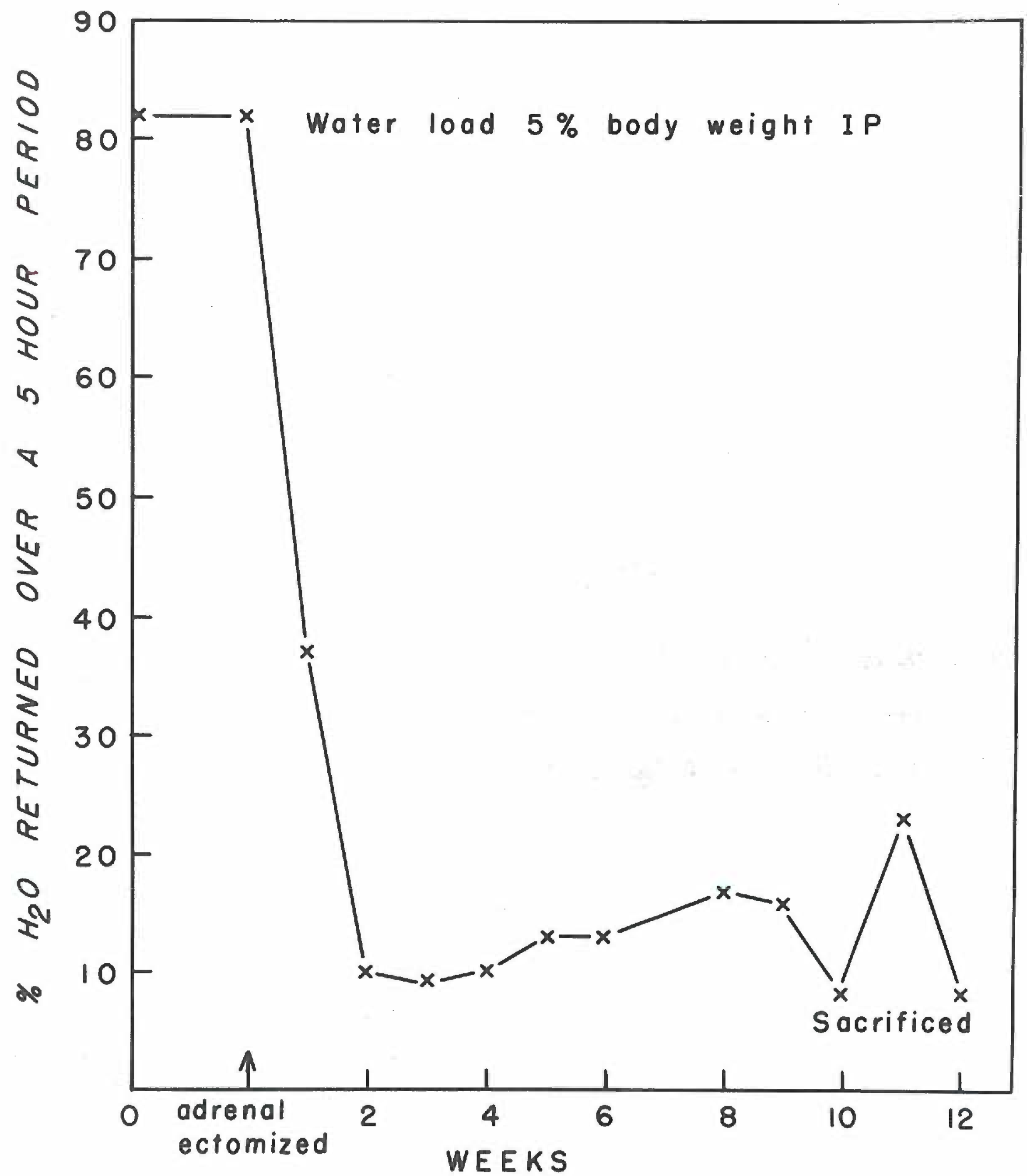
There is also an increase in the absolute number of eosinophils following adrenalectomy. However, inspection of the values on individual animals shows that the increase is not consistent in each animal and therefore, the comparison of absolute eosinophil counts before and after adrenalectomy in a single rat is of no value per se in determining the presence or absence of cortical hormones. The lymphocyte response to stress before and after adrenalectomy did not show consistent change (Table V).

Water tolerance tests were given to 30 chronic adrenalectomized rats for periods of 4 to 15 weeks at 1 or 2 week intervals. Figure V shows the water tolerance tests of a typical chronic adrenalectomized rat. It can be seen that the preoperative control value of 82% fell immediately after adrenalectomy and remained below the positive water tolerance limits of 42% during the 12 week period. With one exception (37% return), water tolerance test values below 31% were consistent in all chronic adrenalectomized animals that did not revert. The presence of accessory adrenal tissue was indicated in the individual rat by tolerance tests of 58 to 117%. Reversion (as indicated by 2 or more consecutive positive water tolerance tests) occurred in 12 operated rats 3 to 9 weeks after adrenalectomy. This figure does not represent all of the possible reversions as 20 of the rats were followed for periods of only 4 to 6 weeks and accessory adrenal tissue might have occurred in some of these animals had they been followed for longer periods of time.

Upon establishing the presence of accessory tissue, the rat was reoperated, by an abdominal approach, the accessory tissue removed, and positive identification of the tissue made by histological examination.

Figure V

Water tolerance tests of a typical chronic adrenalectomized rat followed at 1 and 2 week intervals for 12 weeks.



% H_2O RETURNED OVER A 5 HOUR PERIOD
90
80
70
60
50
40
30
20
10
Water load 5 % body weight IP
Sacrificed
0
adrenal ectomized
2
4
6
8
10
12
WEEKS

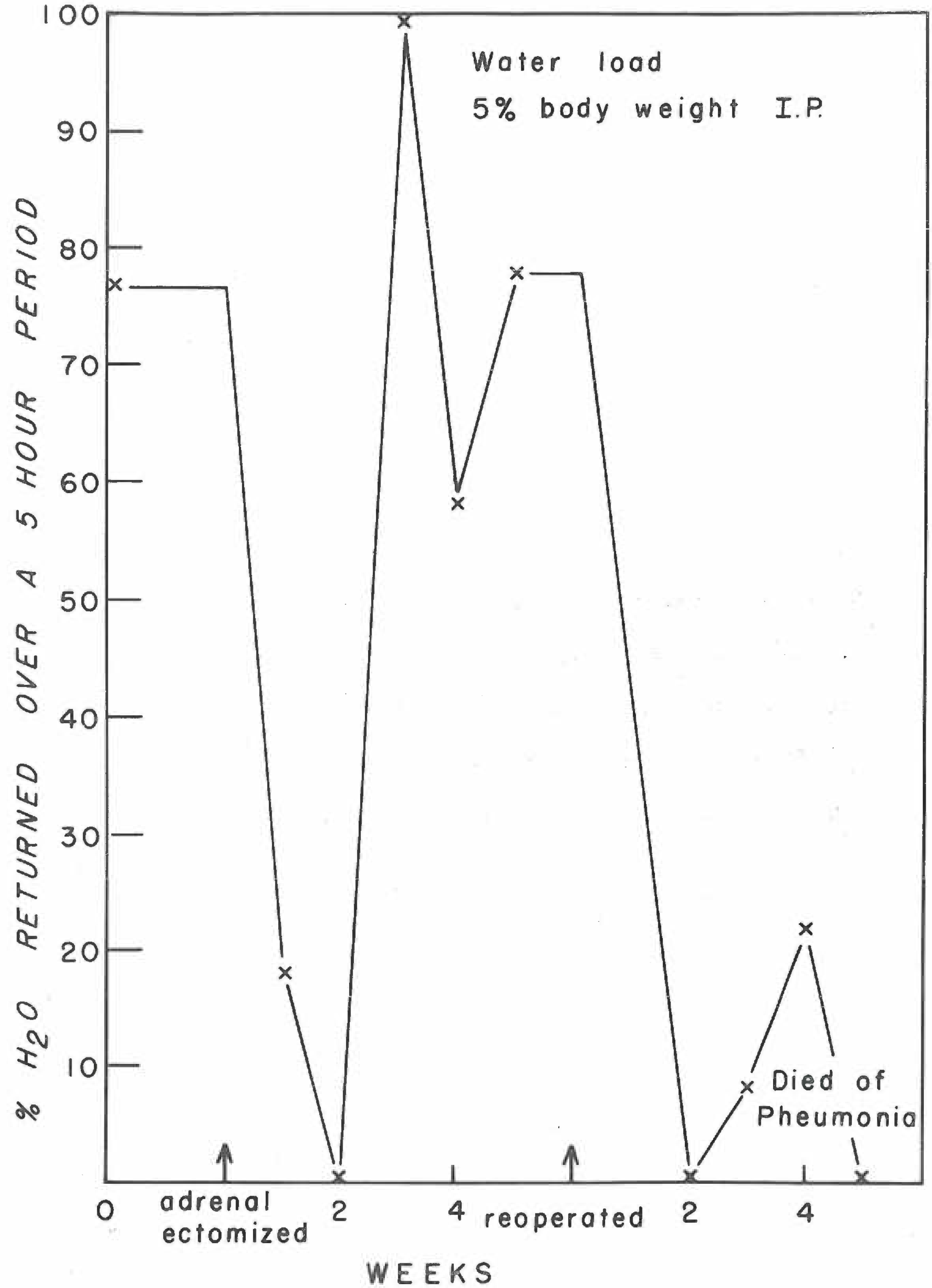
The accessory glands were observed on the dorsal peritoneal wall between the kidney and the diaphragm and occasionally on the aorta in the same area. Water tolerance test values for a typical bilateral adrenalectomized, reverted, and reoperated animal are indicated in Figure VI. This graph shows that 3 weeks after adrenalectomy, three positive water tests were obtained (99%, 58% and 78%). After removal of accessory tissue on reoperation, the water tolerance tests were then negative for four consecutive weeks. Four rats survived reoperation and were followed for 5 to 8 weeks. During this period four water tolerance tests were given each rat and the highest value found was 29% water returned. All rats that died or were sacrificed for analysis were autopsied for accessory adrenal tissue. No adrenal tissue was found in rats showing negative water tests, whereas adrenal tissue was always found in animals with positive tests.

In three adrenalectomized rats, positive water tolerance tests were followed by 1 to 3 weeks of negative tests. In each case, full reversion occurred after that time. The appearance of a single positive water test must be taken, therefore, as an indication of impending reversion. Figure VII shows the water tolerance tests of a typical animal of this type, in which a positive test was indicated 4 weeks after adrenalectomy (42%). This was followed by two negative tests, (0% and 8%). However, reversion was established by the next three positive tolerance tests, (69%, 97% and 93%).

The retention of cortical hormone effects for periods of 1 to 7 days following adrenalectomy has been suggested by experiments on water diuresis by Gaunt and his associates[(61) (75)]. A comparison of water tolerance

Figure VI

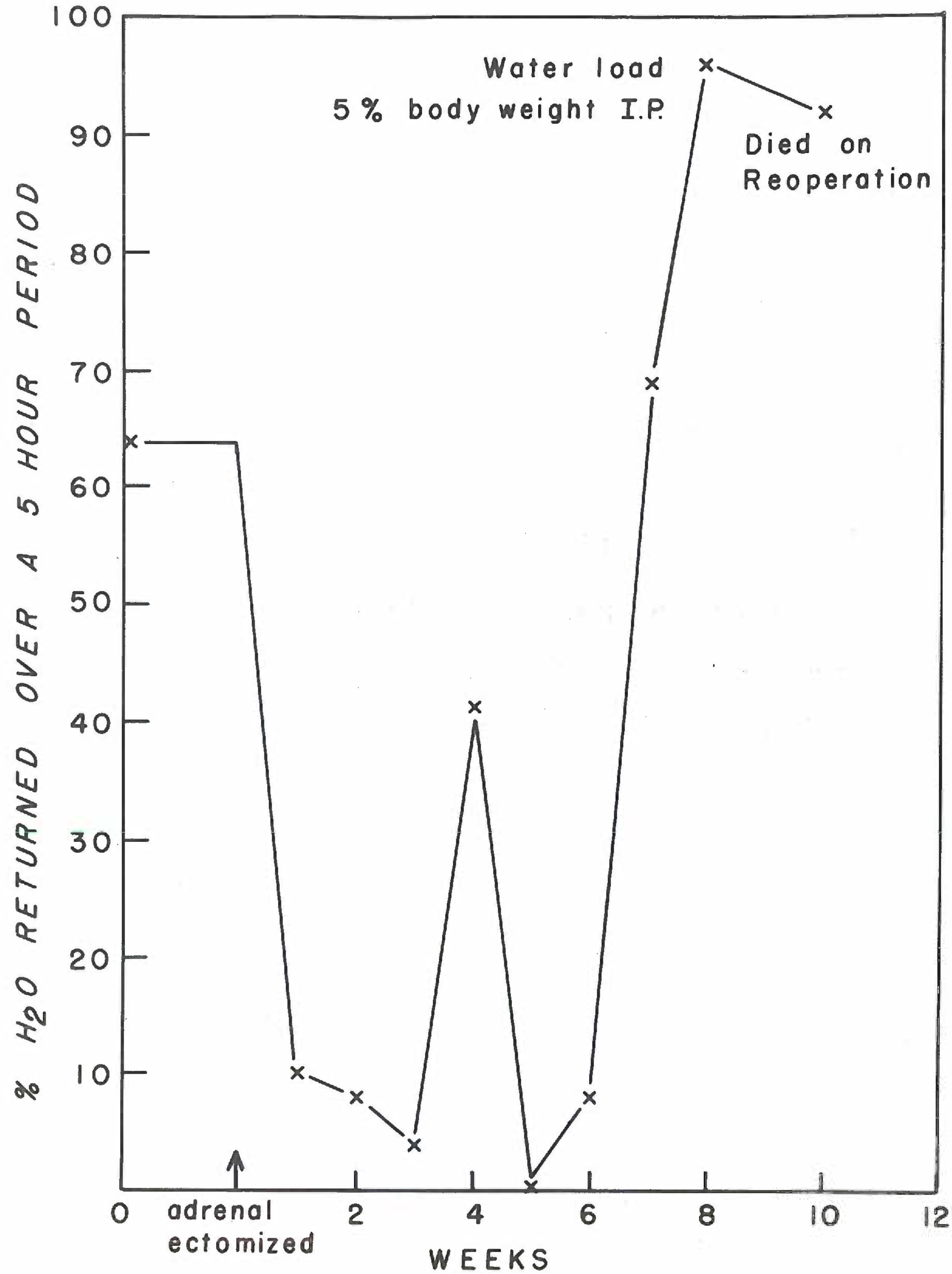
Water tolerance tests of a typical chronic adrenalectomized rat that reverted 3 weeks postadrenalectomy as indicated by the 3 positive water tests. Upon reoperation, the next 4 water tolerance tests were negative.



Water load
5% body weight I.P.
% H_2O RETURNED OVER A 5 HOUR PERIOD
100
90
80
70
60
50
40
30
20
10
Died of Pheumonia
0
adrenal ectomized
2
4
reoperated
2
4
WEEKS

Figure VII

Showing that the appearance of a single positive water tolerance test, when followed by 1 - 3 weeks of negative tests is an indication of impending reversion.



Water load
5 % body weight I.P.
Died on
Reoperation
100
90
80
70
60
50
40
30
20
10
% H_2O RETURNED OVER A 5 HOUR PERIOD
0
adrenal
ectomized
2
4
6
8
10
12
WEEKS

tests in the control rat and in the 4 and 10-day postadrenalectomized animal (Table VI) shows that 11 of the 12 4-day rats had a higher rate of excretion than the 10-day animals. Four of the 12 rats tested on the 4th day after operation showed values in the control range indicating the presence of the effects of cortical hormones. These rats tested 10 days postoperatively gave water tests indicating the absence of functioning adrenal tissue. Impending reversion is not necessarily indicated by a high 4-day test because 3 weeks later the water return may be below 30%. Therefore, the reliability of results on the adrenalectomized rat 4 days or less after bilateral adrenalectomy may be questioned because the animal may not be completely deficient in the effects of adrenal cortical hormones.

Eosinophil and lymphocyte responses to stress were found to be of no value in determining the presence or absence of accessory adrenal tissue in the chronic adrenalectomized animal. Dumm and Ralli[47] have found that in 5-day postadrenalectomized rats, the injection of ACTH (2U/100g) caused both increases and decreases in the number of circulating eosinophils. Also, these workers noted that the injection of cortisone (2mg/100g) to 17-day postadrenalectomized rats caused an increase of circulating eosinophils in 2 of 13 rats, rather than the expected decrease. Padawer and Gordon[76] have reported an unexplained rise in peripheral eosinophils in 2 of 13 adrenalectomized rats given cortisone. In the chronic adrenalectomized rats in this study, eosinophil response to stress determined at varying times from 2 to 12 weeks postoperatively generally correlated to the water tolerance test results, that is, eosinophilia was usually found when the water tests were negative. However, in those

Table VI

The Water Tolerance Test at Varying Times Following Bilateral Adrenalectomy in the Sprague Dawley Rat

Percentage of water returned over a 5-hour period

Control	Adrenalectomized				
	4 days	10 days	3 weeks	4 weeks	5 weeks
99	41	22	28	9	sacrificed
70	50	18	0	0	sacrificed
52	23	25	0	-	67*
76	32	12	0	0	sacrificed
77	65	0	8	22	0
85	25	0	-	4	sacrificed
90	19	0	-	8	sacrificed
77	19	0	-	71	96*
110	42	8	-	96	93*
91	35	23	-	-	17
73	35	14	0	23	0
80	50	17	14	13	16

*Reverted animals. Adrenal tissue found on reoperation.

rats in which the water tolerance test indicated impending or complete reversion, the eosinophil test often failed to detect the presence of accessory tissue. As found in previous experiments, the lymphocyte response again was less reliable than the eosinophil response. From these observations, it appears that the water tolerance test is a more sensitive method of detecting the presence of circulating cortical hormones than are the eosinophil and lymphocyte responses to stress. Although no comparison was made between the water tolerance test and the mortality following salt withdrawal method, a poor correlation between the two methods might be found. The withdrawal of salt constitutes a major stress to the rat and the presence of small amounts of cortical hormones secreted by the regenerating tissue might not be sufficient to maintain electrolyte balance. As a consequence, the animal dies, presumably showing the absence of accessory adrenal tissue. Furthermore, the appearance of a single positive water tolerance test followed by 2 or 3 negative tests (Figure VII) is suggestive of intermittent production of cortical hormones. This may also account for the erratic responses of eosinophils and lymphocytes to stress agents in the chronic adrenalectomized rat.

II. Phosphate Fractions and Creatine in Muscle Tissue of Adrenalectomized and Control Rats.

Having established the validity of the water tolerance test for the presence or absence of functioning adrenal cortical tissue, two series of adrenalectomized animals were prepared. One group of 16 adrenalectomized rats served as a control series. A second series of 11 adrenalectomized rats was given glycocyamine plus betaine. All of the adrenalectomized rats were found to be without the effects of circulating cortical hormones as indicated by the water tolerance test at the time of analysis. A third series of animals consisted of 43 control intact rats which were analyzed before, during and after the adrenalectomized rats.

The results of the analyses of muscle tissue from the 43 intact rats are given in Table VII. The average values found were: inorganic phosphate--23.4 mg % P, creatine phosphate--69.1 mg % P, adenosine diphosphate + adenosine triphosphate--41.6 mg % P, creatine--440.8 mg %, and the percent creatine bound as creatine phosphate--67.0%.

Table VII also shows the results of the analyses of the 16 adrenalectomized rats. The following average values were found: inorganic phosphate--24.8 mg % P, creatine phosphate--64.7 mg % P, ADP+ATP--40.8 mg % P, creatine--428 mg %, and percent bound creatine 64.1%. Because the tissues of adrenalectomized rats were slightly hydrated, all the values for adrenalectomized rat tissue given were corrected on the basis of the control dry weight of 24.4%. Attempts were made to control the muscle hydration by varying concentrations of NaCl in the animal's drinking water. Upon decreasing the saline to 0.7% and 0.5%, the hydration became more severe. 2% saline did not improve the hydration more than did the 1% saline and therefore the 1% saline was provided to the adrenalectomized rats.

A comparison of the intact control rats and the adrenalectomized control rats is made in Table VII. From this table, it can be seen that adrenalectomy causes a significant decrease of the creatine phosphate (4.4 mg % P) below that of the control value. There is no difference in the inorganic phosphate, ADP+ATP, total creatine values and per-

Table VII

A Comparison of the Average Values for Inorganic Phosphate, Creatine Phosphate, Adenosine Diphosphate plus Adenosine Triphosphate, Creatine and Percent Creatine Bound as Creatine Phosphate in the Muscle of Intact Control Rats and Adrenalectomized Control Rats

	Intact Control	Adrenalectomized Control	P
No. of Rats	43	16	
Inorganic Phosphate (mg P / 100 g)	23.4 ± 0.4[*]	24.8 ± 0.5	>.1
Creatine Phosphate (mg P / 100 g)	69.1 ± 0.6	64.7 ± 1.1	<.005
ADP + ATP (mg P / 100 g)	41.6 ± 0.5	40.8 ± 1.5	>.1
Creatine (mg / 100 g)	440.8 ± 5.2[1]	428.0 ± 8.0[2]	>.1
% Bound Creatine	67.0 ± 0.8[1]	64.1 ± 1.2	>.1

* Standard Error

1 N = 35

2 N = 15

cent bound creatine.

Table VIII shows the results of analysis of the 11 adrenalectomized rats given glycocyamine and betaine: Inorganic phosphate--21.4 mg % P, creatine phosphate--69.5 mg % P, ADP+ATP--43.1 mg % P, total creatine --421 mg %, percent bound creatine 69.8 %. The results for the glycocyamine-betaine treated adrenalectomized rats are compared to the values on the adrenalectomized control rats in Table VIII. This table shows that the administration of glycocyamine plus betaine caused a significant rise in the creatine phosphate level in the muscle of the adrenalectomized rat (64.7 to 69.5 mg % P). The difference in inorganic phosphate found between the adrenalectomized control and the adrenalectomized treated rats does not necessarily indicate a difference between the two series of animals. Eight of the 11 glycocyamine treated rats were killed during the months of December, January and February, whereas only 5 of the 16 adrenalectomized control series were killed during this same period. When the intact control rats are similarly divided, the 13 inorganic phosphate values for the winter months are significantly lower than the 30 inorganic phosphate values determined over a 2 year period, winter months excepted, $P < .005$. However, creatine phosphate levels did not show such a seasonal variation $P > .1$. Therefore, a difference in inorganic phosphate levels as indicated in Table VIII can be explained on the basis of possible seasonal variation. There was no difference in the total creatine levels of the two series of animals, (428 mg % adrenalectomized control and 421 mg % adrenalectomized treated). There was no change in ADP+ATP, and percent bound creatine levels. It seems probable that the higher levels of CrP found

Table VIII

A Comparison of the Average Values for Inorganic Phosphate, Creatine Phosphate, Adenosine Diphosphate plus Adenosine Triphosphate, Creatine and Percent Creatine Bound as Creatine Phosphate in the Muscle of Adrenalectomized Control Rats and Adrenalectomized Rats Given Glycocyamine-Betaine

	Adrenalectomized Control	Adrenalectomized + Glycocyamine-Betaine	P
No. of Rats	16	11	
Inorganic Phosphate (mg P / 100 g)	24.8 ± 0.5*	21.4 ± 0.6	<.005
Creatine Phosphate (mg P / 100 g)	64.7 ± 1.1	69.5 ± 1.3	<.01
ADP + ATP (mg P / 100 g)	40.8 ± 1.5	43.1 ± 0.7	>.1
Creatine (mg / 100 g)	428.0 ± 8.0[1]	421.0 ± 9.0	>.1
% Bound Creatine	64.1 ± 1.2[1]	69.8 ± 0.9	<.005

* Standard Error

[1] N = 15

in this study as compared to values reported in the literature were due to the in situ technique of freezing muscle, the method of tissue storage and the care exercised in preparing and analyzing the filtrate. Albaum[33] reported average creatine phosphate values of 39 mg % P for similar muscle in his control rats. Helve[30] obtained an average control creatine phosphate value of 15.4 mg % P. Conway and Hingerty[31][32] reported creatine phosphate values in control rats averaging 78 mg % P. However, from inspection of Conway and Hingerty's data, it is apparent that this value includes both the creatine phosphate and inorganic phosphate phosphorus. Therefore, this value is not representative of the creatine phosphate content of the muscle of their experimental animals. The inorganic phosphate levels as found by Albaum (34 mg % P) and Helve (42.5 mg % P) are higher than those reported in this study. This increase in inorganic phosphate was probably due to the extreme lability of creatine phosphate and their inadequate technique in muscle sampling and analysis, resulting in the breakdown of creatine phosphate.

The decrease in muscle creatine phosphate following adrenalectomy also does not agree with the results of Albaum who found no change in this substance upon removal of the adrenals. However, in view of the asthenia found in patients with Addison's disease, a defect in muscle energy production or utilization is probable. Ochoa[77] found lowered creatine phosphate levels in the muscles of adrenalectomized frogs associated with a lowered work performance of these muscles. Helve[30] reported a lowered creatine phosphate in rats after adrenalectomy; however, his animals were not maintained on saline after operation and were killed by immersion in liquid air. The inability to demonstrate differences between the creatine phosphate levels in the muscles of adrenalectomized and of control rats as reported by some workers is probably due to the rapidity with which creatine phosphate degenerates thereby masking actual differences in the muscle levels.

The ability of glycocyamine plus betaine to raise the levels of creatine phosphate in the muscles of adrenalectomized rats rules out the mediation of the adrenals in this process. Because the levels of free creatine are not raised by administration of glycocyamine and betaine, it is doubtful that the main action of this treatment is according to the mechanism outlined previously, that is, increasing the levels of free creatine by providing the synthesizing mechanism with additional supplies of precursors. From the results obtained in this study, the mechanisms by which glycocyamine-betaine increase muscle creatine phosphate levels is not apparent. The total creatine was the same in the muscles of the adrenalectomized and control rats, but glycocyamine-betaine treatment increased the percent of this creatine present as creatine phosphate.

It is interesting to note that the ADP+ATP levels in the muscle tissue were constant in the three series of animals studied (Tables VII and VIII). The physiologically fundamental nature of the creatine phosphate-ATP energy relation to the animal organism is such that the homeostatic mechanisms apparently maintain ATP levels at the expense of the creatine phosphate energy reserves.

SUMMARY

1. A water tolerance test has been developed for the presence of functioning adrenal tissue in the chronic adrenalectomized rat.

2. Eosinophil and lymphocyte responses to ACTH or to stress are not as sensitive as is the water tolerance test in detecting the presence of accessory adrenal tissue.

3. The creatine phosphate level in the muscles of adrenalectomized rats is significantly lower than that of control intact animals.

4. Administration of glycocyamine plus betaine significantly raises the level of creatine phosphate in the adrenalectomized rat; the mediation of the adrenal is apparently not required for this action.

5. There are no significant differences between the levels of total creatine, inorganic phosphate, and adenosine diphosphate plus adenosine triphosphate in the muscles of control intact rats, adrenalectomized rats and adrenalectomized rats given glycocyamine-betaine.

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