ENZYME STUDIES IN GLYCINE-FED STRESSED RATS

by

Russell Ray Mac Donald, B.S.

A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment
of the requirements for the degree of
Master of Science

June 1961

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Carbohydrate storage in the body is influenced to a considerable degree by the amount and the composition of the diet. One significant study in this regard is that of Mirski and co-workers (32) who showed that feeding diets rich in carbohydrate led to increased liver glycogen storage in rats, while a high protein diet had little effect in normal animals. However, following a twenty-four hour fast or a cold stress of several hours, the rats previously fed a high protein diet had increased liver glycogen compared to rats prefed a high carbohydrate diet. Mirski and co-workers (32) termed this phenomenon of increased liver glycogen storage following stress in animals prefed a diet high in protein as the "protein effect." This observation of the "protein effect" has been further confirmed by Guest (13) in 1941 and by Neuburger and Brown (34) in 1942. Todd and co-workers have amplified these findings on the "protein effect" in a series of papers. (6,42,43,45,46).

Todd, Barnes and Cunningham (45) reported prefeeding rats a diet containing ten percent of the amino acid glycine and obtained liver glycogen levels after a twenty-four hour fast, some three times that found in rats prefed a control diet lacking the added glycine. After a twenty-four hour fast rats prefed diets containing L-leucine or L-glutamic acid, failed to show any change in liver glycogen storage compared to rats prefed the control diet; although, D,L-alanine prefeeding did show a slight increase of glycogen storage. The increased liver glycogen levels following a twenty-four hour fast in rats prefed glycine was termed the "glycine effect" by Todd and co-workers (45)

This term seems preferable to "protein effect" since the later work involves feeding extra amounts of glycine and not of protein.

The "glycine effect" now refers to the increased glycogen storage after stress, and the increased glycogen synthesis during recovery after stress in rats, as a result of prefeeding a diet containing ten percent of added glycine.

Cunningham, Barnes and Todd (6) employed a combination of a fast and a rather large dose of insulin as the stress condition. In these experiments the glycine-fed animals showed ten times as much liver glycogen as the control-fed rats at five hours following insulin administration. Also, the depression of blood sugar was much less marked in the glycine-fed animals, leading to the conclusion that the extra carbohydrate in the glycine-fed animals could not be explained on the basis of a reduced rate of glycogenolysis in these animals.

Ringer and Lusk (35) found that injested glycine was quantatively excreted as urinary glucose in dogs made glucosuric with phlorhizin. This indicated that a possible pathway of glycine metabolism in the body was its conversion to carbohydrate material. However, Olsen, Hemingway and Nier (35) reported an increased liver glycogen level sixteen hours after feeding glycine, labeled with C^{13} in the carboxyl group, to mice, but the glycogen contained only one percent of the isotope fed. These data indicate that the effect of glycine on the glycogen stores in indirect.

Talman and Todd (46) determined the free and total glycine and total protein contents of various body tissues in rats prefed either the control or the glycine diet. Total protein contents of the various

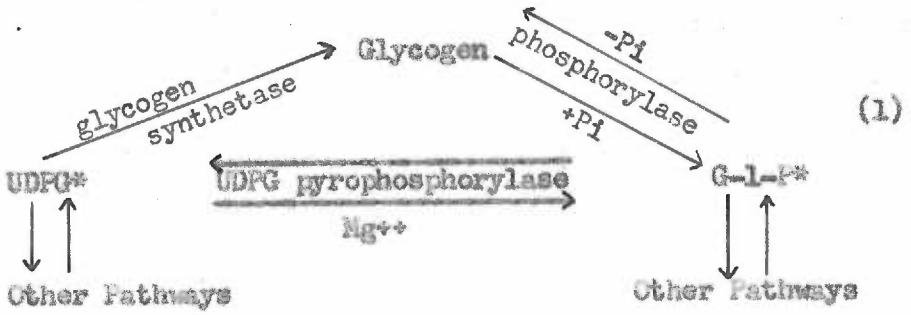
tissues studied did not differ between the two diets while in kidney, liver, muscle, blood, and intestine, increased amounts of free glycine were found in rats prefed glycine, and the blood and intestine also showed some of the increased glycine content to be in a bound form. Calculations were outlined from the data presented and from data obtained in the insulin experiments, indicating that animals prefed the glycine diet have in their bodies, after an eight hour fast and five hours of insulin action, about six times as much carbohydrate as can be accounted for by direct conversion to carbohydrate of the extra glycine present before the action of insulin.

Hess and Shaffron (16) studied the glyconeogenetic action of glycine in fasted rats. They gave the amino acid by stomach tube and determined liver glycogen levels at various post feeding periods. Two peaks of glycogen formation were observed, the first, although very small, was found at one hour after the glycine feeding and was considered to represent direct conversion of glycine to glycogen. The second peak, found at fourteen hours after glycine feeding, was considered to constitute evidence of an indirect effect, i.e., glyconeogenesis from a source other than glycine.

Talman and Todd (42,46) found that nitrogen retention was greater during the first twenty-four hours in rats fed the glycine diet, while during a twenty-four hour fast, nitrogen excretion was greater in rats prefed the glycine diet. These data provide additional evidence that glycine effects carbohydrate storage, not through a direct conversion of injested glycine to glycogen, but through increasing glyconeogenesis from other sources.

Todd and Allen (44) reported that adrenalectomized rats failed to elicit the "glycine effect", but when such animals were maintained with hydrocortisone (1 mg hydrocortisone per 100 g body weight twice a day for two days) the "glycine effect" was clearly evident. At the present time it appears that adrenal cortical hormone, stress, and glycine prefeeding are required to demonstrate this phenomenon. It is likewise clear, however, that extra cortical hormone is not a requisite.

In recent years biochemical studies have clearly shown the vast majority of chemical reactions within the living organism to be enzymatically controlled. Glycogen is no exception as evidenced by the actions of the enzymes phosphorylase and glycogen synthetase. The general glycogen cycle may be summarized by the following scheme:



The two enzymes of interest here are phosphorylase and glycogen synthetase. Both enzymes require the presence of glycogen for activity and both are involved with α-1,4-glucosyl linkages of the terminal glucose units in glycogen chains. Leloir and Goldemberg (23) have used differential centrifugation of fresh tissue homogenates to show that both phosphorylase and glycogen synthetase followed the glycogen fraction, and appeared to be very intimately bound to the glycogen molecules.

---

* UDPG = uridine diphosphoglucose, G-1-P = glucose-1-phosphate, Pi = inorganic phosphate

Muscle phosphorylase differs from most enzymes by having two distinct forms, an active and an inactive molecule. Phosphorylase has optimum activity at pH 6.8 and optimum temperature between 37 to 39°C. The active form of phosphorylase is quite often referred to as phosphorylase-a and according to Green and Cori (12), has a molecular weight of around 340,000 to 400,000, but more recent reports indicate a molecular weight of about 495,000 (19). The function of phorphorylase is to catalyze reversibly the transfer of glucose units between glucose-1-phosphate (G-1-P) and the terminal glucose unit on glycogen. In this reaction inorganic phosphate (Pi) is either combined with a terminal glucose unit to form G-1-P or is released as the glucose is incorporated into the glycogen molecule. A specific ratio of Pi to G-1-P of less than 3.5 is necessary for phosphorylase to catalyze the formation of glycogen from G-1-P in vitro. Since analyses show that muscle tissue has a ratio of around 275 (26) it seems unlikely that phosphorylase synthesizes glycogen in vivo. More likely, the enzyme normally degrades glycogen.

Phosphorylase activity determinations utilize the reversibility of the phosphorylase reaction by decreasing the ratio of Pi to G-1-P to below 3.5, thus causing the enzyme to synthesize glycogen. In this way a realiable determination of phosphorylase activity is available by measuring the amount of Pi before and after the enzymatic action.

Muscle phosphorylase-a is inactivated by phosphorylase-rupturing enzyme or prosthetic-group removing enzyme (PR enzyme) forming two molecules of phosphorylase-b, each of which seems to be equivalent to about half of the phosphorylase-a molecule. The molecular weight is about

242,000. Conversely, the two molecules of phosphorylase-b are converted to one molecule of phosphorylase-a by the action of phosphorylase-b kinase. These two reactions may be summarized as follows:

$$\text{phosphorylase-a} \xrightarrow{\text{Pr enzyme}} 2\ \text{phosphorylase-b} + 4\ \text{Pi} \qquad (2)$$

and,

$$2\ \text{phosphorylase-b} + 4\ \text{ATP}^{*} \xrightarrow{\text{phosphorylase-b kinase}} \text{Phosphorylase-a} + 4\ \text{ADP}^{*} \qquad (3)$$

Phosphorylase-b has an absolute dependence upon the presence of adenylic acid (adenosine monophosphate - AMP) for in vitro activity. Phosphorylase-b, in the presence of AMP has been shown by Cori and co-workers (4,5) to have essentially the same enzyme kinetics as phosphorylase-a. In the presence of AMP it is possible to determine from one enzyme preparation, total phosphorylase activity, phosphorylase-a activity and the inactive fraction which can be obtained by subtraction. The action of AMP on the phosphorylase molecules appears to lower the energy of activation (5). A combination of AMP with phosphorylase has been demonstrated by ultracentrifuge and spectrophotometric studies. Ultracentrifuge studies (20,31) utilizing different ratios of phosphorylase to AMP, demonstrated breaks in sedimentation and diffusion curves indicating that two molecules of AMP bound one molecule of phosphorylase-b, while four molecules of AMP bound one molecule of phosphorylase-a. These investigators (20,31) utilizing a spectrophoto-

---

* ATP adenosine triphosphate, ADP adenosine diphosphate

metric shift in the ultraviolet peaks also demonstrated the binding of AMP with the two different phosphorylase molecules.

A further example of the catabolic nature of phosphorylase is found in studies on glycogen storage diseases in humans (15,21,22,23, 24,25,33,39). In these muscle diseases there is a normal or increased amount of glycogen present, and a total lack or an extremely small amount of phosphorylase activity, indicating the requirement of the enzyme for glycogen degradation.

The presence in tissues of the enzyme responsible for anabolism of glycogen was first reported by Leloir and Cardini (27) in 1957. This observation has been amply confirmed (1,14,24,26,28,29,37,39). Later Leloir and Goldemberg (28) named this glycogen forming enzyme, glycogen synthetase (uridine diphosphoglucose-glycogen transglucosylase). Glycogen synthetase functions by catalyzing the transfer of glucose from UDPG (uridine diphosphoglucose) to a terminal glucose of an α-1,4-glucosyl linkage of glycogen. This enzyme has been shown to have a rather unphysiologic optimum pH between 8 and 8.5 and an optimum temperature of 36 to 39°C. With UDPG as substrate the Michaelis constant (K_m) for glycogen synthetase is about 5×10^{-4} M (28,29). Enzyme characteristics for glycogen synthetase are similar regardless of the tissue from which it is isolated.

Radioisotope studies (28,29,37) utilizing C^{14}-glucose in UDPG* have shown that during the action of glycogen synthetase, the C^{14}-glucose is quantatively transferred to glycogen. In some of these test systems UDP (uridine diphosphate, UDPG after loss of glucose) was measured. Comparable enzyme activities were found using either the

UDP released or the isotopic activity of glycogen as end points.

In the elucidation of the characteristics of glycogen synthetase from liver, a heat and acid stable activator was found. This activator proved to be glucose-6-phosphate (G-6-P) (29). The activation of glycogen synthetase by G-6-P was highly significant since the activity was increased 4 to 15 fold. Half maximal increase in enzyme velocity was attained at extra G-6-P concentrations of 6 X 10^{-4} M (29). The specific function or action of G-6-P is still unknown, although it was suggested (28) that a "protection" effect is afforded the enzyme so as to lessen any inactivation by other constituents within the assay system. Other phosphorylated sugars such as galactose-6-phosphate and fructose-6-phosphate have shown a definite "protection" action similar to G-6-P, but to a lesser extent. This "protective" action of G-6-P is more pronounced in highly purified glycogen synthetase preparations than in crude enzyme preparations, although G-6-P is without effect during storage of highly purified enzyme even when stored at -15°C for twenty-four hours.

Similarities between phosphorylase and glycogen synthetase are comparable in two significant respects; glycogen is a common substrate and cysteine is a common activator or "protector". Cysteine has a small, consistant action ("protective" ?) on phosphorylase even in crude extracts, while the action on glycogen synthetase is small or absent in crude extracts, but is very pronounced (up to 50 percent increase in activity) in highly purified systems. Glycogen synthe-

tase is insensitive to the presence of G-1-P, AMP, ATP, galactose-1-phosphate, or insulin at low concentrations, while sometimes these compounds have a drastic effect upon the activity of phosphorylase. Epinephrine lacks any effect on glycogen synthetase except in very high, unphysiological concentrations (37) (from 5 to 50 ug per ml) while the effect of even small amounts is very pronounced on active phosphorylase. This action is through activation of phosphorylase-b kinase which in turn forms phosphorylase-a from phosphorylase-b.

As shown by the "glycine effect", the constituents of a diet may have a very marked effect upon metabolic pathways. On the supposition that 10 percent glycine in the diet of rats may cause increased blood and tissue ammonia, the possibility of a "toxcity" that might effect some enzyme systems and not others was considered. Ammonia toxcity in rats has been partially alleviated by the administration of arginine (38). Under experimental conditions outlined in this thesis, feeding arginine failed to influence the typical "glycine effect." Creatine is a metabolic product of arginine and was prefed as an addition to the glycine and control diets in order to determine if it influenced the "glycine effect."

EXPERIMENTAL METHODS

Male Sprague-Dawley rats weighing 150 to 250 g were used in all experiments. The weights of the animals on the control or the experimental diets were matched at the start of each experiment.

Four experimental diets were employed; control diet, control diet with added creatine, glycine diet, and glycine diet with added creatine. The compositions of the control and the glycine diets are shown in Table 1.

Table 1

Composition of Glycine Diet and Control Diet

	Control Ration Percent	Glycine Ration Percent
Casein*	16	16
Yeast**	10	10
Salt Mixture***	5	5
Cottonseed Oil****	5	5
Cod Liver Oil	2	2
Dextrose*****	8	8
Dextrin*	54	44
Glycine	0	10

* Nutritional Biochemicals Corporation
** Squibb (flavored yeast)
*** Nutritional Biochemicals Corporations (Salt Mix W)
**** Wesson Oil
***** Mallinckrodt (anhydrous)

The two basic diets shown in Table 1 differ only in their content of dextrin and glycine. The other two synthetic diets were prepared by adding two percent creatine to each of the above rations. The two basic diets were designed to give a total of twenty percent protein, supplied by the casein and the yeast. This amount of protein allows satisfactory growth for semi-synthetic rations. After thoroughly mixing the dry ingredients, the Wesson Oil and the Cod

Liver Oil were incorporated to yield a homogenous mixture.

The handling of the animals may conveniently be described under three categories, A) pre-experimental, B) experimental, and C) post-experimental.

A) The pre-experimental or stock colony animals were placed in individual cages upon arrival from the breeders. These animals were weighed each day in order to accustom them to frequent handling, and to assure that the animals were healthy as far as could be ascertained by physical examination and by weight gain.

B) The experimental handling of the rats consisted of placing them on control ration for one day to accustom them to synthetic diets. At the end of the first twenty-four hour conditioning period the animals were matched as to weights, and then placed in the desired group and fed one of the appropriate experimental diets. After eighteen hours on the experimental diet, the food was removed, weighed, and the animals weights recorded. A final meal was divided and given at two different times. The first part of the meal, received by all animals was given immediately after completion of the previous eighteen hours; this part was two g of the control diet containing all ingredients except glycine, dextrin, and creatine. The second part of the final meal was given two hours later by stomach tube; and was composed of a solution of dextrin only, glycine plus dextrin, dextrin plus creatine or all three ingredients. It was designed to equalize food and caloric intake among the animals of an individual experiment, and represented an amount of these substances equivalent

to that contained in two g of the diet under study. Four hours following the stomach tubing, at which time absorption is essentially complete, some of the animals were sacrificed and others subjected to stress.

The stress employed in all of the work reported here consisted of swimming the rats in 14°C water for three 10 minute periods with 30 minute "rest" periods between swims at room temperature. The animals were wet, cold, and shivering during these 30 minute intervals out of the water, which made the stress a total of one and one-half hours. Some of the animals were sacrificed immediately and are referred to as stressed animals, while others were allowed to recover at room temperature for an additional three hours before they were sacrificed. These animals are referred to as recovery animals.

It has been previously established in this laboratory that more consistent glycogen levels are obtained by sacrificing the animals in the morning hours. Therefore, the experiments were arranged so that the animals were started on the stress, or non-stressed rats sacrificed, at a time between 7:00 and 8:30 A.M.

C) The post-experimental treatment concerns sacrificing, and handling of the tissues after death. All animals were uniformally killed by an intraperitoneal injection of 0.6 to 0.8 ml of Nembutal. When deep anesthesia was attained the right gastrocnemius muscle and the liver were removed and assayed for glycogen by the method of Good, Kramer and Somogyi (11). The glucose was estimated by the Shaffer-Hartmann method using the Somogyi modi-

fication (40). Following the removal of the liver, the skin of the left rear leg was stripped back and held with a hemostat. The two hind legs were stretched and tied around a wire frame. The entire posterior end of the rat and the wire frame were emersed in a dry ice-ethanol cold bath (-70 to -78°C) for four to five minutes. After the legs were well frozen, the entire left leg was removed from the body of the rat, wrapped in aluminum foil and stored in a dry ice chest surrounded by dry ice for the determination of enzyme activities at a later date.

Enzyme Determinations

Phosphorylase activity. The following reagents were used in the determination of active phosphorylase (phosphorylase-a) and of total phosphorylase.

1. Glycerophosphate buffer: 0.06 M sodium betaglycerophosphate in 0.4 M sodium fluoride, pH 7.0 at room temperature, was stored at 3 to 5°C.
2. Cysteine: 0.06 M cysteine solution (free base from Nutritional Biochemicals Corporation). The reagent was stored at 3 to 5°C and prepared fresh biweekly.
3. Glucose-1-phosphate--glycogen--adenylic acid reagent: 45 mM glucose-1-phosphate (G-1-P), and 2.1 mM muscle adenylic acid (adenosine monophosphate--AMP) in 1.3 percent (w/v) glycogen solution, pH 6.8 at room temperature, was stored at -15 to -20°C.
4. Glucose-1-phosphate--glycogen reagent: 45 mM glucose-1-phosphate (G-1-P) in a 1.3 percent (w/v) glycogen solution, pH

6.8 at room temperature, was stored at -15 to -20°C.

5. Homogenizing medium: 0.001 M EDTA solution (ethylenediamine tetraacteic acid). The reagent was stored at 3 to 5°C.

6. Elon: 1 percent Elon (para methylaminophenyl sulphate) in 3 percent sodium bisulfite. The reagent was stored at room temperature and prepared fresh weekly.

7. Molybdate--sulfuric acid: 5.0 g of $Na_2MoO_4 \cdot 2H_2O$ was dissolved in water, 19 ml of concentrated sulfuric acid was added and the mixture diluted to 1.0 liter. The reagent was stored at room temperature.

Phosphorylase assays were made by the method of Illingworth and Cori (17) with slight modifications as follows: The frozen leg was removed from the dry ice chest and the outer layers of tissue discarded. About 350 mg of the muscle tissue in the area of the gastrocnemius was then sliced directly into a tared, ground glass homogenising tube and reweighed to determine the amount of tissue. Cold EDTA homogenizing medium was added in the ratio of 1.0 ml of solution to 100 mg of tissue. The muscle was homogenized with a ground glass pestle for approximately one minute, transferred to a stoppered plastic centrifuge tube and centrifuged at 2000 X g (3100 rpm using a 269 head in an IEC refrigerated centrifuge) for ten minutes at -2 to -4°C. While centrifugation was proceeding, 0.5 ml of both glucose-1-phosphate--glycogen--adenylic acid reagent and glucose-1-phosphate--glycogen reagent were preincubated at 37°C. Adenylic acid (AMP) was present only in the preincubation tube in which total phosphorylase was to be assayed, for in the absence of AMP, active phosphorylase is

assayed, while in the presence of AMP, total phosphorylase is assayed. The reactions involved here are:

$$\begin{array}{l} X\ (G\text{-}1\text{-}P) \\ + \\ \text{glycogen}\text{---}(\text{glucose})_n \end{array} \xrightarrow[AMP]{\text{phosphorylase-a+b}} \begin{array}{l} \text{glycogen}\text{---}(\text{glucose})_{n+x} \\ + \\ X\ (Pi) \end{array} \qquad (4)$$

also,

$$\begin{array}{l} X(G\text{-}1\text{-}P) \\ + \\ \text{glycogen}\text{---}(\text{glucose})_n \end{array} \xrightarrow{\text{phosphorylase-a}} \begin{array}{l} \text{glycogen}\text{---}(\text{glucose})_{n+x} \\ + \\ x\ (Pi) \end{array} \qquad (5)$$

These reactions are both reversible but with a ratio of inorganic phosphate (Pi) to G-1-P of less than 3.5, both reactions will proceed as indicated.

After the muscle homogenate had been centrifuged, 0.5 ml of the supernatant fluid was added to 2.0 ml of the original EDTA homogenizing medium and mixed; 1.0 ml of the diluted supernatant was then mixed with 2.0 ml of glycerophosphate buffer solution. A 2.0 ml portion of this enzyme-buffer solution was mixed with an equal volume of fresh cysteine reagent. A 0.5 ml aliquot of the enzyme preparation was then added to the preincubated mixture of G-1-P and glycogen, with and without AMP, and mixed. The pH of the final reaction mixture was 6.8 at 37°C. A 0.2 ml aliquot of the reaction mixture was removed at 0, 4, and 8 minutes. The enzymatic activity was stopped immediately by heating for one minute in a boiling water bath.

The inorganic phosphate (Pi) released in the reaction was determined by the method of Fiske and Subbarow (9) modified by Gomori (10) as follows: 3.0 ml of molybdate—sulfuric acid reagent and 0.5 ml of Elon were added to the samples and thoroughly mixed. The intensity

of blue color which develops was read on a Beckman D.U. spectrophotometer as μg of phorphorus released per minute per g of tissue.

Glycogen synthetase activity. The following reagents were used in the determination of glycogen synthetase activity.

1. Sucrose—EDTA homogenizing medium: Isotonic 0.25 M sucrose in 0.001 M EDTA (ethylenediamine tetraacetic acid), adjusted to pH 8.5 at room temperature, was stored at 3 to 5°C.
2. UDPG: UDPG (uridine diphosphoglucose). 5 mM UDPG solution (Sigma—sodium salt $\cdot 5H_2O$, 98 to 100 percent pure). The reagent was stored at -15 to -20°C.
3. G-6-P: G-6-P (glucose-6-phosphate). 1 mM G-6-P solution (Sigma—disodium salt$\cdot 3H_2O$, 98 to 100 percent pure). The reagent was stored at -15 to -20°C.
4. Glycine buffer: 15 mM glycine solution, adjusted to pH 8.5 at room temperature, was stored at -15 to -20°C.
5. EDTA solution: 30 mM EDTA solution (ethylene diamine tetraacetic acid), adjusted to pH 8.5 at room temperature, was stored at -15 to -20°C.
6. Phosphoenolphyruvic acid: 10 mM phosphoenolpyruvic acid (as the tri-cyclohexylammonium salt, A grade, assay: 97.6 percent, from California Biochemical Research) in 0.4 M potassium chloride. The reagent was stored at -15 to -20°C.
7. Glycogen solution: 4.8 percent (w/v) glycogen solution (Nutritional Biochemicals Corporation). The reagent was stored at -15 to -20°C.
8. Dinitrophenylhydrazine: 0.1 percent dinitrophenylhydrazine

in 2 N hydrochloric acid. The dinitrophenylhydrazine was dissolved in hot acid and diluted to volume. The reagent was stored at room temperature and prepared fresh every two weeks.

9. 10 N sodium hydroxide: The reagent was stored at room temperature.

10. UDP standard: 1.6 mM standard UDP solution (uridine diphosphate—Sigma, disodium salt·$5H_2O$, 96 to 98 percent purity). The reagent was stored at -15 to -20°C.

11. Pyruvate kinase: Pyruvate kinase (muscle, in ammonium sulfate suspension, Nutritional Biochemicals Corporation) diluted 1:20 with 0.1 M magnesium sulfate. The diluted reagent was stored at 3 to 5°C and maintained its activity for several weeks.

Glycogen synthetase was assayed by a slightly modified method of Leloir and Goldemberg (24). The frozen leg was removed from the dry ice storage chest and the outer layers of tissue discarded. About 300 mg of tissue in the area of the gastrocnemius was sliced directly into a tared, ground glass homogenizing tube and reweighed to determine the weight of the tissue. Cold sucrose—EDTA homogenizing medium was added in the ratio of 1.0 ml to each 100 mg of tissue. The tissue was homogenized with a ground glass pestle for approximately one minute, transferred to a plastic centrifuge tube and centrifuged at 2000 X g for ten minutes at -2 to -4°C. While centrifuging the homogenate the following reagents were mixed and preincubated at 37°C: 0.10 ml of

UDPG, 0.10 ml of G-6-P, 0.10 ml of EDTA, 0.10 ml of glycine buffer, and 0.10 ml of glycogen. 0.10 ml of the centrifuged supernatant was mixed with the preincubated reaction mixture and 0.10 ml aliquots were removed at 0, 3, and 5 minutes. In this way the 0 time sample served as a blank for the following samples and the amount of UDP (uridine diphosphate) in this blank was later subtracted from the following samples. The enzymatic action was stopped immediately by heating the samples for one minute in a boiling water bath. The reaction,

$$\begin{array}{l} x\ (\text{UDPG}) \\ + \\ \text{glycogen—(glucose)}_n \end{array} \xrightarrow{\text{glycogen snythetase}} \begin{array}{l} \text{glycogen—(glucose)}_{n+x} \\ + \\ x\ (\text{UDP}) \end{array} \qquad (6)$$

releases UDP as one product. The UDP is then converted to UTP with phosphoenolpyruvate by the reaction.

$$\begin{array}{l} \text{UDP} \\ + \\ \text{phosphoenolpyruvic acid} \end{array} \xrightarrow{\text{pyruvate kinase}} \begin{array}{l} \text{UTP} \\ + \\ \text{pyruvic acid} \end{array} \qquad (7)$$

The pyruvic acid is then determined by the method of Lu as modified by Kachmar and Boyer (18). Thus, the final assay procedure from the initial reaction (reaction 6) was as follows: 0.05 ml of phosphoenol-pyruvic acid was added to the reaction mixture containing the reaction product, UDP (reaction 7). To the reaction tube was added 0.05 ml of pyruvate kinase and the mixture incubated for 15 minutes at 37°C. After 15 minutes of incubation, 0.30 ml of dinitrophenylhydrazine was added, and the mixture incubated at 37°C for an additional five minutes. 0.4 ml of 10 N NaOH was added to produce a yellow-brown color of dinitro-phenylhydrazone according to the reaction:

(8)

dinitrophenylhydrazine + pyruvic acid → dinitrophenylhydrazone of pyruvic acid + water

(8)

2.2 ml of 95 percent ethanol was added to the vessel to precipate the glycogen present and the samples were centrifuged for 15 minutes at 1500 rpm. The intensity of the clear yellow-brown chromagen was read in a Beckman D.U. spectrophotometer at 520 mμ. The optical density recordings obtained from the samples were compared with those from a standard curve prepared by the method outlined using from 0.016 μM to 0.16 μM of UDP. The activity of glycogen synthetase was expressed as μM of UDP released per minute per g of tissue.

RESULTS

The "glycine effect", as previously defined, refers to the increased glycogen storage after stress, and the increased glycogen synthesis during recovery after stress in rats, as a result of prefeeding a diet containing 10 percent of added glycine. The purpose of this investigation was to determine whether certain enzymes concerned with glycogen metabolism exhibited an alteration in activity prior to, during, or after stress with different diets.

In this report all glycogen determinations in either the muscle or liver were carried out by other workers in this laboratory as a part of another program. In Figures 1 through 6 data are not available for times other than zero time (non-stress), 1.5 hours stress and after 3 hours of recovery after stress. Although curves were drawn connecting these points, intervening values are entirely unknown. Table 2 and Figure 1 illustrate both numerically and graphically the glycogen levels of liver and skeletal muscle of rats prefed the glycine diet or the control diet. From the data presented in Table 2 the "glycine effect" is clearly evident except in muscle following one and one-half hours of stress. Similarily, Table 2 and Figure 2, show numerically and graphically the glycogen levels when creatine had been added to the prefed glycine and control diets. Creatine feeding abolished the typical "glycine effect" in the liver. In muscle, however, the "glycine effect" showed clearly after stress but the data indicate a "blocking" of the phenomenon following recovery after stress. Also of interest is the fact that liver glycogen levels before stress are considerably lower in creatine fed

animals on either the control or the glycine diet (Table 2). The same can be said for the muscle glycogen values although the differences (with and without creatine) are far less marked.

Total and active muscle phosphorylase activities are presented in Table 3 and Figure 3 for the glycine fed and control fed rats, while Table 4 and Figure 4 presents the data from animals prefed creatine-glycine diet and creatine-control diet. Total phosphorylase activities failed to show any significant differences regardless of the stress condition or the prefed diet. Active phosphorylase, regardless of diet, was always increased in activity in non-stress animals compared to the stressed animals. A slight decrease in activity was observed in the recovery animals compared to the stressed rats. All values from non-stress animals were significantly lower in phosphorylase-a activity compared to the stressed and recovery animals with one exception. This exception was between the glycine fed recovery rats and the creatine-control fed non-stress animals. The slight decrease in phosphorylase-a activity from stress to recovery was significant only in the case of the creatine-glycine fed animals.

Significant differences of total phosphorylase and active phosphorylase for all conditions outlined herein are summarized in Table 5. An aid to understanding these data is provided by bracketing groups of animals that do not differ among themselves but do differ from groups of animals outside of the enclosing brackets. The Duncan test

for analysis of variance (8) at the 5 percent level was used in all statistical treatment of phosphorylase activities.

Glycogen synthetase activities of tissues from rats prefed glycine and control diets are presented in Table 6 and Figure 5. With animals on these diets, significant differences in activity were present only in the non-stress rats. Conversely, Table 7 and Figure 6 show significant glycogen synthetase activity differences only in the recovery animals when creatine was present in the glycine and control diets. Increased activity was found in creatine-control fed animals compared to creatine-glycine fed animals following recovery after stress, but not before or immediately after stress. In no instance was a significant difference in enzyme activities observed between animals on any one diet but under different stress conditions. However, significant differences between animals fed the various diets with any one specific stress condition are summarized in Table 8. In Table 8, as in Table 5, all groups of animals within a bracket, do not differ among themselves, but do differ from those outside of the bracket. The t test of Student at the 5 percent level was used for all statistical analyses of glycogen synthetase activities.

Table 2.

Muscle and Liver Glycogen Concentrations of Animals Fed All Diets.

	Animal Status					
	Non-Stress		1.5 Hour Stress		3.0 Hour Recovery	
	Muscle	Liver	Muscle	Liver	Muscle	Liver
Glycine Diet	0.52** ±0.02*	3.00 ±0.16	0.11 ±0.02	1.85 ±0.03	0.21 ±0.03	2.38 ±0.10
Control Diet	0.56 ±0.03	3.33 ±0.26	0.09 ±0.01	0.79 ±0.08	0.13 ±0.01	0.76 ±0.11
P	>10%	>10%	>10%	<1%	<1%	<1%
Creatine-glycine Diet	0.47 ±0.02	1.58 ±0.13	0.14 ±0.02	1.29 ±0.11	0.22 ±0.02	0.55 ±0.13
Creatine-control Diet	0.42 ±0.03	1.03 ±0.09	0.07 ±0.01	1.25 ±0.19	0.20 ±0.02	0.51 ±0.10
P	>10%	>10%	<1%	>10%	>10%	>10%

**Mean of data from 10 animals. Glycogen levels expressed as percent wet weight.

*Standard error of mean.

Table 3

Phosphorylase Activities of Muscle.

Animals Fed Glycine or Control Diet.

	Animal Status					
	Non-Stress		1.5 Hour Stress		3.0 Hour Recovery	
	Total	Active	Total	Active	Total	Active
Glycine Diet	3513** ±250*	125 ±30	3413 ±350	1575 ±200	3488 ±280	1186 ±310
Control Diet	3936 ±310	208 ±40	3531 ±430	2059 ±210	3551 ±380	1441 ±310
P	>5%	>5%	>5%	>5%	>5%	>5%

**Mean of data from 10 animals. Phosphorylase activities expressed as µg P released per minute per g tissue.

*Standard error of mean.

Table 4

Phosphorylase Activities of Muscle.

Animals Fed Creatine-glycine or Creatine-control Diet.

	Animal Status					
	Non-stress		1.5 Hour Stress		3.0 Hour Recovery	
	Total	Active	Total	Active	Total	Active
Creatine-glycine Diet	3454** ±290*	238 ±120	3741 ±280	2198 ±310	3442 ±330	1500 ±240
Creatine-control Diet	4003 ±280	493 ±260	3661 ±380	2443 ±350	3884 ±390	2294 ±440
P	>5%	>5%	>5%	>5%	>5%	<5%

**Mean of data from 10 animals. Phosphorylase activities expressed as μg P released per minute per g tissue.

*Standard error of mean.

Table 5

Significant Phosphorylase Activities at the 5% Level as Determined by the Duncan Test of Analysis of Variance. Animals within Brackets do not Differ while Animals outside of any One Bracket Differ from Those within the Bracket.

TOTAL PHOSPHORYLASE

Non-stress Animals, Creatine-control Fed
Non-stress Animals, Control Fed
3.0 Hour Recovery Animals, Creatine-control Fed
1.5 Hour Stress Animals, Creatine-glycine Fed
1.5 Hour Stress Animals, Creatine-control Fed
3.0 Hour Recovery Animals, Control Fed
1.5 Hour Stress Animals, Control Fed
Non-stress Animals, Glycine Fed
3.0 Hour Recovery Animals, Glycine Fed
Non-stress Animals, Creatine-glycine Fed
3.0 Hour Recovery Animals, Creatine-glycine Fed
1.5 Hour Stress Animals, Glycine Fed

ACTIVE PHOSPHORYLASE

1.5 Hour Stress Animals, Creatine-control Fed
3.0 Hour Recovery Animals, Creatine-control Fed
1.5 Hour Stress Animals, Creatine-glycine Fed

1.5 Hour Stress Animals, Control Fed
1.5 Hour Stress Animals, Glycine Fed
3.0 Hour Recovery Animals, Creatine-glycine Fed
3.0 Hour Recovery Animals, Control Fed

3.0 Hour Recovery Animals, Glycine Fed

Non-stress Animals, Creatine-control Fed

Non-stress Animals, Creatine-glycine Fed
Non-stress Animals, Control Fed
Non-stress Animals, Glycine Fed

Table 6

Glycogen Synthetase Activities of Muscle.

Animals Fed Glycine or Control Diet.

	Animal Status		
	Non-Stress	1.5 Hour Stress	3.0 Hour Recovery
Glycine Diet	0.9990* ±0.0400**	1.0200 ±0.0310	1.0370 ±0.0270
Control Diet	1.1000 ±0.0180	1.1080 ±0.0310	1.0790 ±0.0340
P	< 5%	> 5%	> 5%

*Mean of data from 10 animals. Glycogen synthetase activities expressed as μM UDP released per minute per g tissue.

**Standard error of mean.

Table 7

Glycogen Synthetase Activities of Muscle.

Animals Fed Creatine-glycine or Creatine-control Diet.

	Animal Status		
	Non-stress	1.5 Hour Stress	3.0 Hour Recovery
Creatine-glycine Diet	1.0170* ±0.0280**	0.9810 ±0.0360	0.9740 ±0.0330
Creatine-control Diet	1.0260 ±0.0170	1.0270 ±0.0300	1.0340 ±0.0230
P	> 5%	> 5%	< 5%

*Mean of data from 10 animals. Glycogen synthetase activities expressed as μM UDP released per minute per g tissue.

**Standard error of mean.

Table 8

Statistically Significant Glycogen Synthetase

Activities at the 5% level as Determined by the t test.

Animals within Brackets do not Differ while Animals outside

of any One Bracket Differ from Those within the Bracket.

Non-stress	1.5 Hour Stress	3.0 Hour Recovery
Control Diet	Control Diet	Control Diet
Creatine-control Diet	Creatine -control Diet	Creatine-control Diet
Glycine Diet	Glycine Diet	Glycine Diet
Creatine-glycine Diet	Creatine-glycine Diet	Creatine-glycine Diet

Figure 1

Muscle and Liver Glycogen Levels, Percent Wet Weight.

All Animals Prefed Glycine or Control Diet.

Each Point Represents the Mean of Data from 10 Animals.

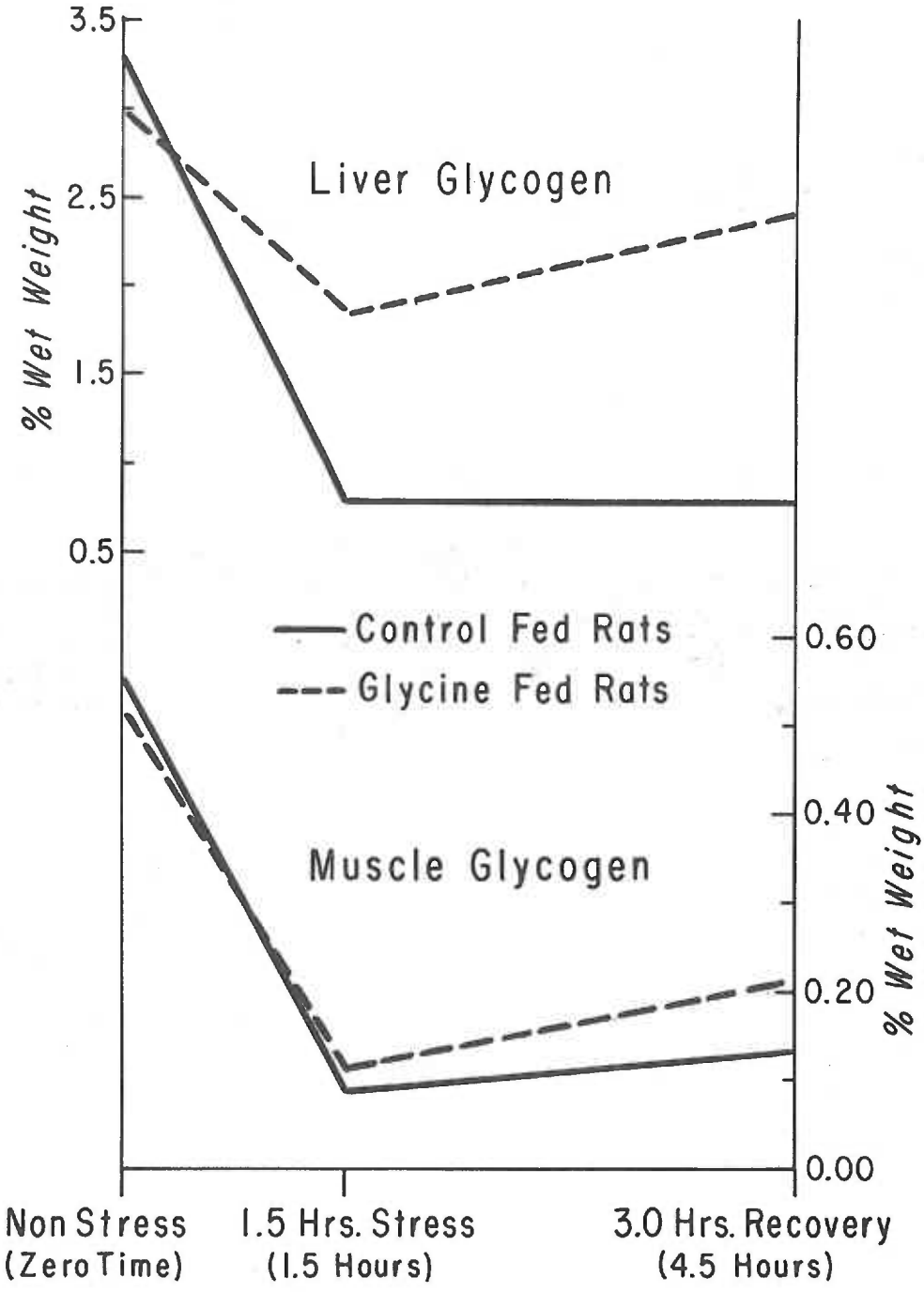

3.5
2.5
1.5
0.5
% Wet Weight
Liver Glycogen
Control Fed Rats
Glycine Fed Rats
Muscle Glycogen
0.60
0.40
0.20
0.00
% Wet Weight
Non Stress
(Zero Time)
1.5 Hrs. Stress
(1.5 Hours)
3.0 Hrs. Recovery
(4.5 Hours)

Figure 2

Muscle and Liver Glycogen Levels, Percent Wet Weight.

All Animals Prefed Either Creatine-glycine

or Creatine-control Diets.

Each Point Represents the Mean of Data from 10 Animals.

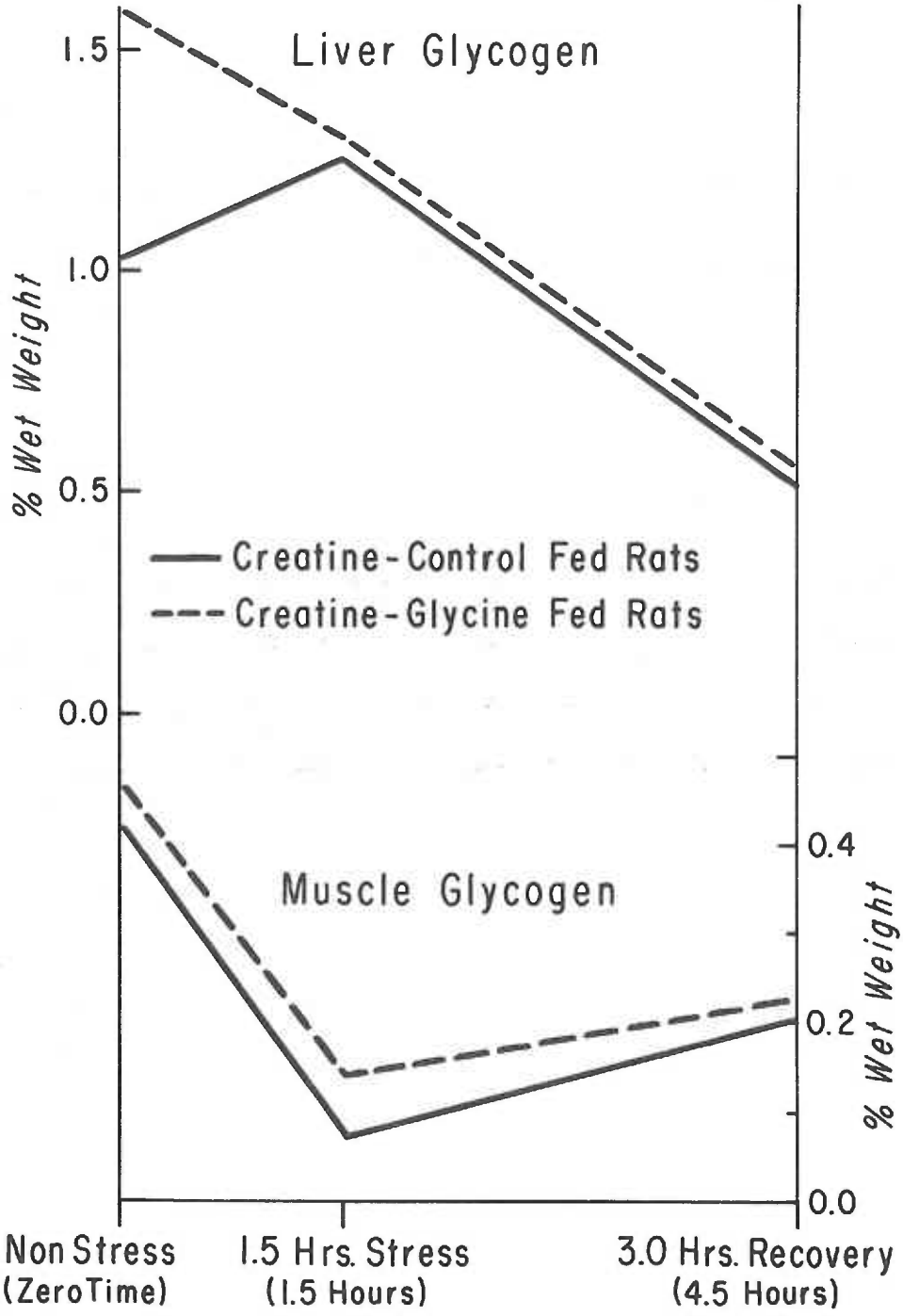

Liver Glycogen
1.5
1.0
0.5
0.0
% Wet Weight
Creatine-Control Fed Rats
Creatine-Glycine Fed Rats
Muscle Glycogen
0.4
0.2
0.0
% Wet Weight
Non Stress
(ZeroTime)
1.5 Hrs. Stress
(1.5 Hours)
3.0 Hrs. Recovery
(4.5 Hours)

Figure 3

Muscle Phosphorylase Activities,

1 Unit = µg P Released per Minute per g Tissue.

All Animals Prefed Either Glycine or Control Diet.

Each Point Represents the Mean of Data from 10 Animals.

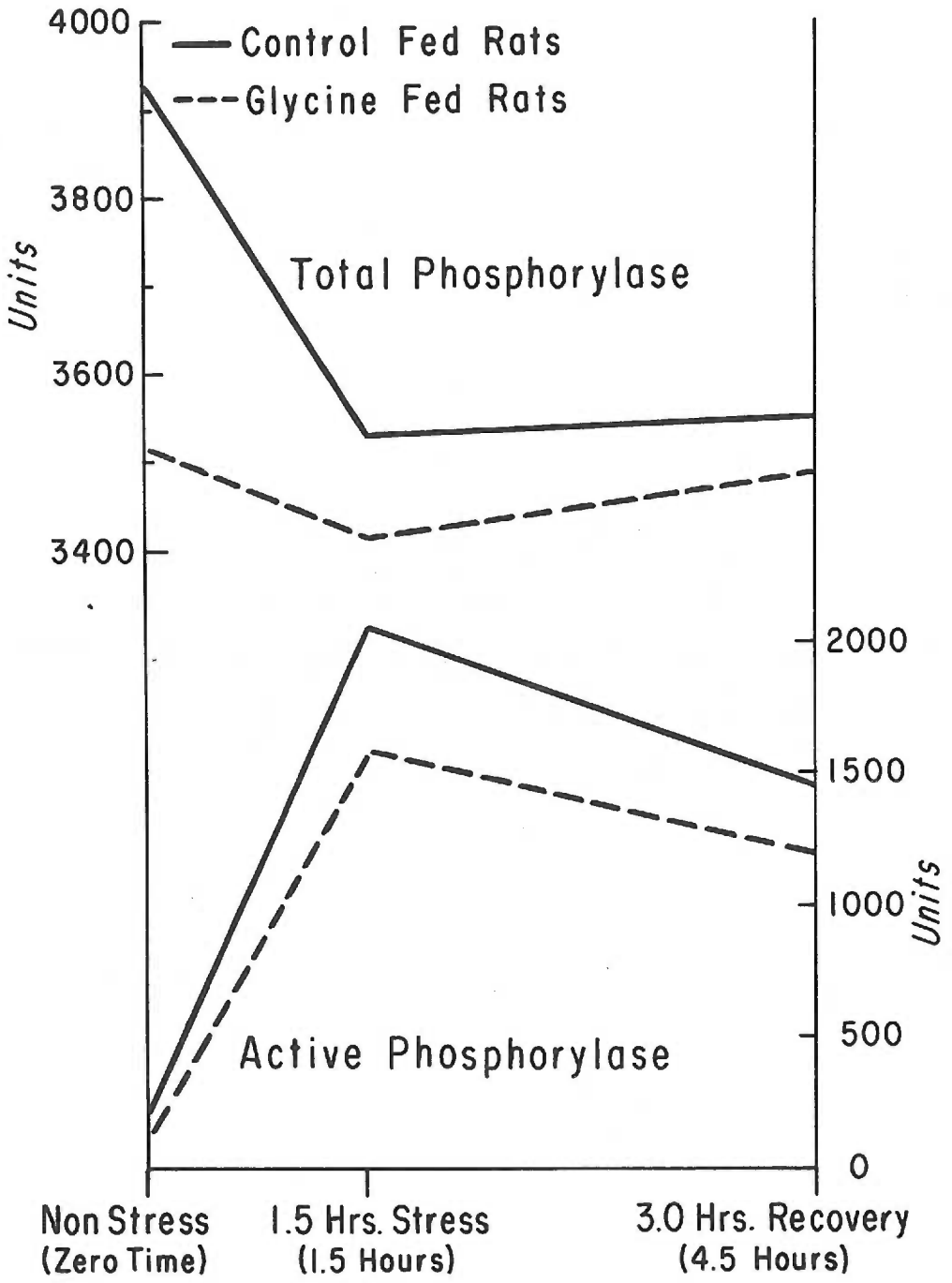

4000
3800
3600
3400
Units
Control Fed Rats
Glycine Fed Rats
Total Phosphorylase
2000
1500
1000
500
0
Units
Active Phosphorylase
Non Stress
(Zero Time)
1.5 Hrs. Stress
(1.5 Hours)
3.0 Hrs. Recovery
(4.5 Hours)

Figure 4

Muscle Phosphorylase Activities,

1 Unit = µg P Released per Minute per g Tissue.

All Animals Prefed Either Creatine-glycine

or Creatine-control Diet.

Each Point Represents the Mean of Data from 10 Animals.

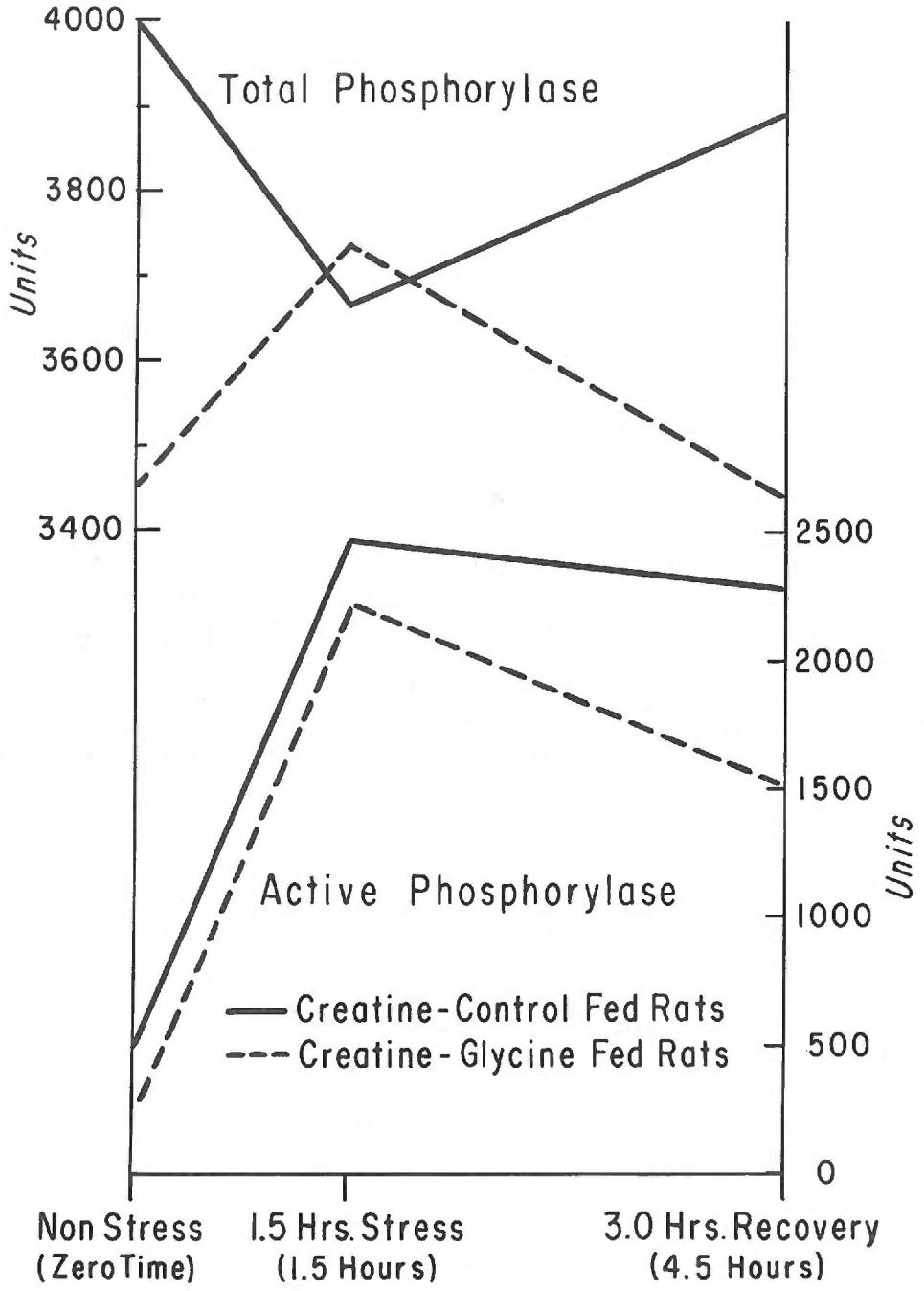

4000
3800
3600
3400
Units
Total Phosphorylase
2500
2000
1500
1000
500
0
Units
Active Phosphorylase
Creatine-Control Fed Rats
Creatine-Glycine Fed Rats
Non Stress (Zero Time)
1.5 Hrs. Stress (1.5 Hours)
3.0 Hrs. Recovery (4.5 Hours)

Figure 5

Muscle Glycogen Synthetase Activities,

1 Unit = μM UDP Released per Minute per g Tissue.

All Animals Prefed Either Glycine or Control Diet.

Each Point Represents the Mean of Data from 10 Animals.

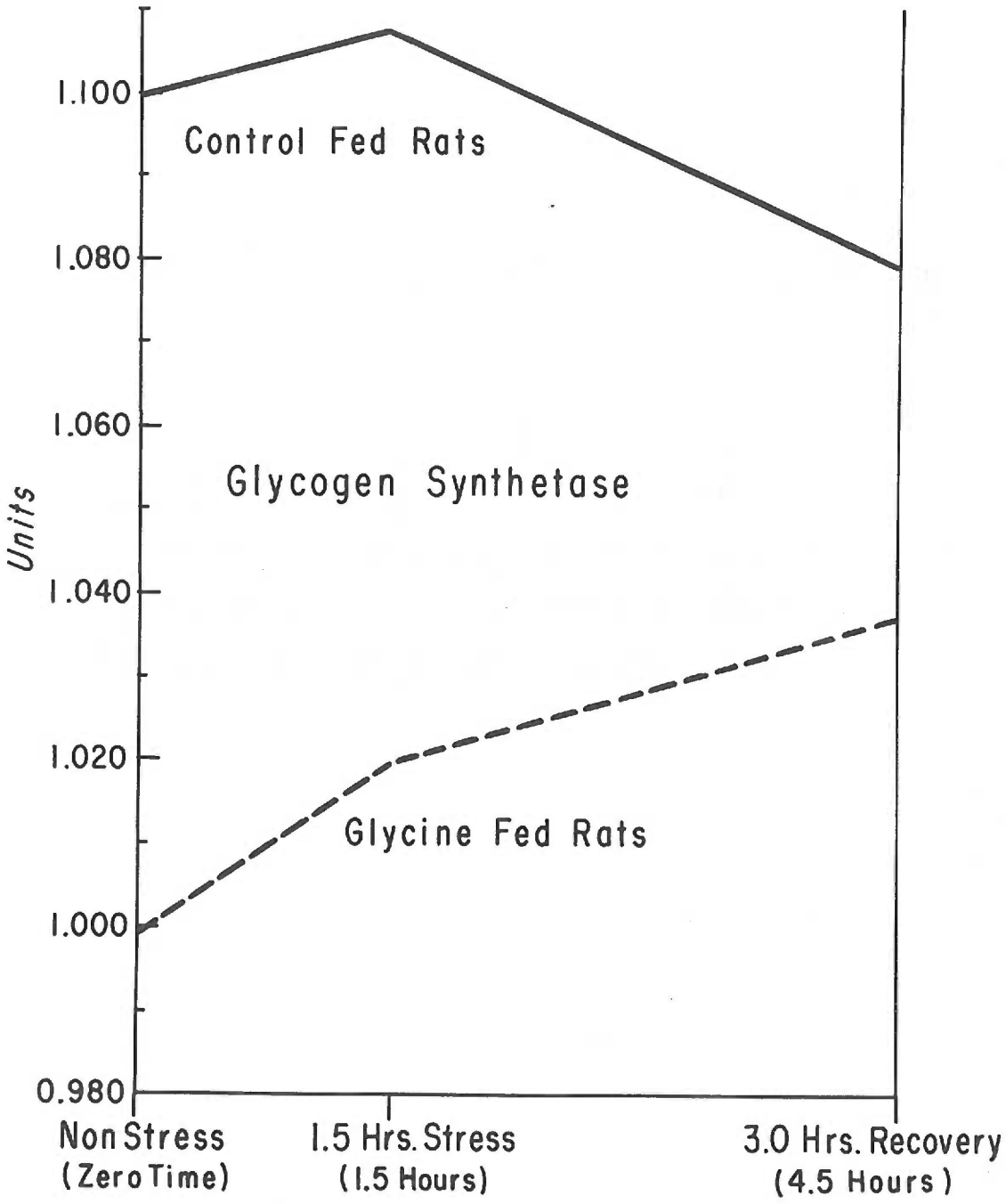

1.100
1.080
1.060
1.040
1.020
1.000
0.980
Units
Control Fed Rats
Glycogen Synthetase
Glycine Fed Rats
Non Stress
(Zero Time)
1.5 Hrs. Stress
(1.5 Hours)
3.0 Hrs. Recovery
(4.5 Hours)

Figure 6

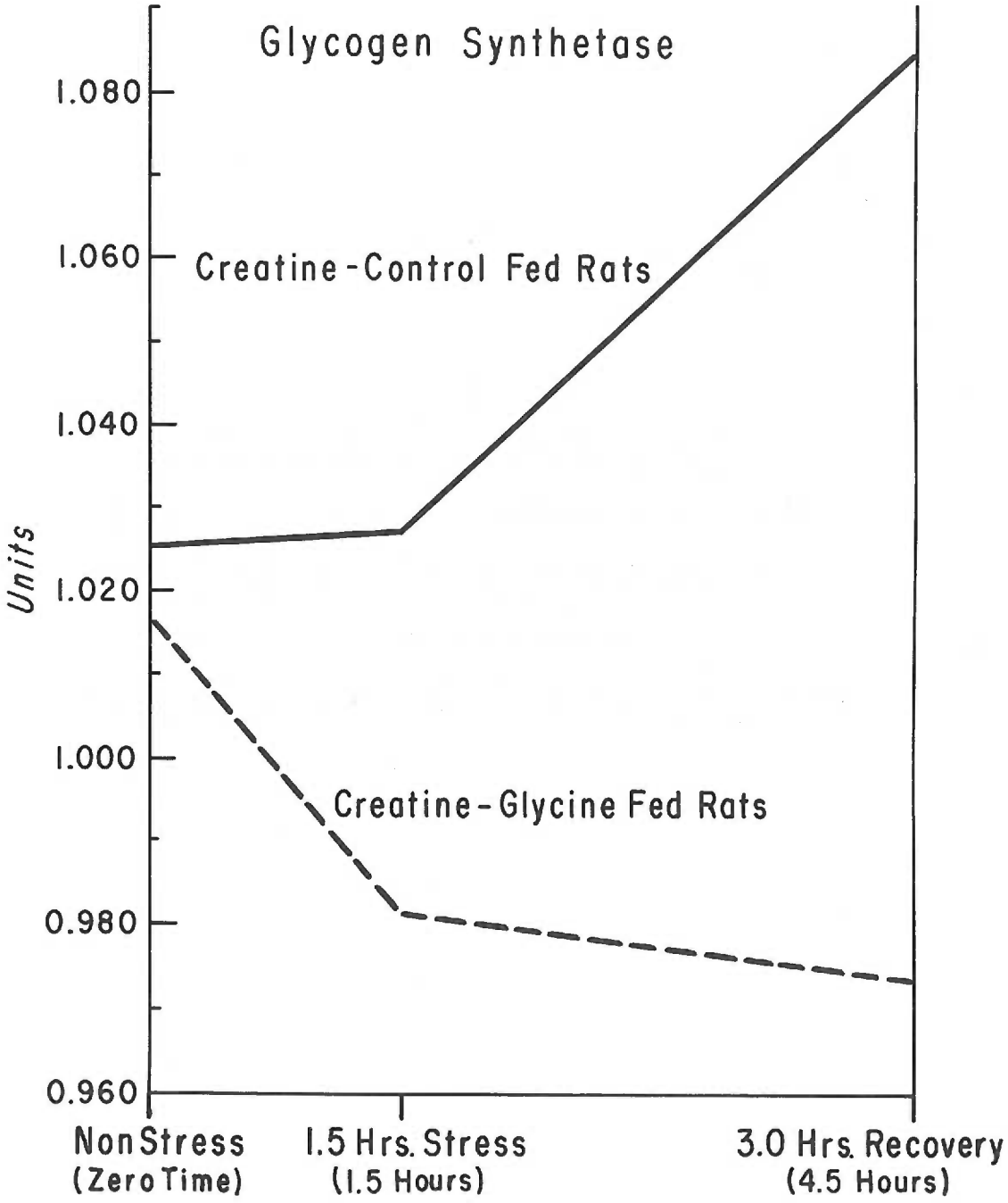
Muscle Glycogen Synthetase Activities,

1 Unit = μM UDP Released per Minute per g Tissue.

All Animals Prefed Either Creatine-glycine

or Creatine-control Diet.

Each Point Represents the Mean of Data from 10 Animals.


Glycogen Synthetase
1.080
1.060
1.040
1.020
1.000
0.980
0.960
Units
Creatine-Control Fed Rats
Creatine-Glycine Fed Rats
Non Stress
(Zero Time)
1.5 Hrs. Stress
(1.5 Hours)
3.0 Hrs. Recovery
(4.5 Hours)

DISCUSSION

One of the purposes of this investigation was to determine if the activities of enzymes phosphorylase and glycogen synthetase were altered by different diets or by stress. Alteration of enzyme activity or concentration has been referred to as enzyme induction or repression in various bacterial investigations and more recently in mammalian studies (21). In rats it has been shown that a variety of liver enzymes are inducible (increased activity) with stress or injections of hydrocortisone (2,7,23). These inducible enzymes are tryptophan pyrrolase, fructose-1,6-diphosphatase, and glucose-6-phosphatase. The reverse of enzyme induction is repression or decreased enzyme activity. Thus, in the present experiments, if an altered enzyme activity appeared in the case of either total phosphorylase or glycogen synthetase, then enzyme induction or repression would be suggested. The data presented in Tables 3 and 4 appear to eliminate induction or repression of phosphorylase because total phosphorylase activity was completely independent of the diets used or the stress imposed upon the animals.

Enzyme induction and repression may be involved in the case of glycogen synthetase since this enzyme showed differences in activity with diet and at different times in the stress procedure. Glycogen synthetase activity of non-stress control fed rats was significantly greater than any of the non-stress animals prefed the other diets containing glycine, creatine, or creatine plus glycine. This trend of

glycogen synthetase activity corresponds roughly with the glycogen levels found in muscle of these rats in the non-stress condition. Under these conditions the muscle glycogen levels of the creatine fed, the glycine fed or the creatine plus glycine fed animals remained below those of the control fed rats. This suggests a repression of glycogen synthetase activity with the long term (24 hours) feeding of the supplements. Enzyme induction or repression with any one specific diet in the short time involved in the stress was not apparent, as evidenced by the lack of significant differences in activity at any time in the stress procedure. Animals prefed the creatine-control or the creatine-glycine diets showed opposite trends in glycogen synthetase activity. The non-stress and stressed animals on the two diets did not differ statistically in enzyme activity, but after recovery the creatine-control fed rats had significantly higher activity than the creatine-glycine fed animals. The data are indicative of enzyme induction but are far from conclusive. This can be visualized by reference to Figures 5 and 6 in which it is seen that the increased activity (induction ?) in the creatine-control fed animals is about equivalent to the decreased activity (repression ?) of the creatine-glycine fed animals following recovery after stress. If glycogen synthetase activities were indicative of the glycogen stores within the muscle and liver, then the animals prefed the glycine diet (and exhibiting the "glycine effect") should have had considerably greater activities than those prefed the control diet. Also, in the "blocked" "glycine effect", the animals prefed the creatine-control or the creatine-glycine diets should have about the same glycogen synthetase

activity because the muscle glycogen stores are about equivalent. But, glycogen synthetase activities do not follow the glycogen stores in the muscle as shown by an inverse relationship between enzyme activities of the "glycine effect" animals, and the animals fed creatine with or without glycine in which instances the "glycine effect" was "blocked." Such animals showed almost equal muscle glycogen levels after recovery while the glycogen synthetase activities were significantly greater in the creatine-control fed rats compared to the creatine-glycine fed rats. Thus, there appears to be little correlation between enzyme activities and glycogen levels in the muscle.

Knox (21) found that maximum induction of tryptophan pyrrolase occurred in his rats at about two and one-half hours after the injection of hydrocortisone or of tryptophan. Therefore, if glycogen synthetase were capable of induction or repression, and as rapidly as in the case of tryptophan pyrrolase, then it would seem reasonable to assume that with the time intervals used in the experiments reported here, enzyme induction or repression would be evident. When the "glycine effect" was evident, significant differences in muscle enzyme activities were absent; when the "glycine effect" was "blocked" by feeding creatine in the diets, significant differences in enzyme activity were present. Thus, the activities of this enzyme offer no help in explaining the mechanism of the "glycine effect." The data presented by Leloir and co-workers (29) indicate that in rats, muscle has the greatest glycogen synthetase activity of any tissue in the body. This suggests that muscle has enough enzyme to meet ordinary stress requirements placed upon it.

As previously mentioned, significant differences in total phosphorylase (sum of phosphorylase-a or active and phosphorylase-b or inactive) activity were not found regardless of the diet or the stress employed. It is not established that phosphorylase-a values obtained on frozen tissue represent in vivo enzyme activity. However, since all the values presented here were on frozen tissues from animals treated differently, differences in activity, should represent valid variations.

An important consideration is the amount or the activity of phosphorylase-a present at any time during the experiment. The non-stress animals were below 500 units of active phosphorylase (a low level) regardless of the diet prefed, and such values can be considered to represent resting muscle. These data confirm the findings of Krebs and Fisher (22) that the majority of the phosphorylase in resting muscle is in the form of phosphorylase-b and not, as previously held by Cori (3) in the form of phosphorylase-a.

During the stress, with a concomitant increase in energy demand, an increased phosphorylase-a activity might be expected since the enzyme supplies glucose from stored glycogen. The recovery animals would be expected to have phosphorylase-a activities quite similar to those found in non-stress animals because the recovery animals were dry, warm, inactive, and with the exception of being tired, were apparently in good physical condition. This supposition was definitely not the case. Data in Tables 3 and 4 show greater muscle phosphorylase-a activities following stress and recovery after stress, regardless of diet.

Theoretically, it would appear that as long as the glucose produced from glycogen was needed by the tissues and phosphorylase-a was present, glycogen would be continually broken down until the tissue was devoid of it. The limitation here is that phosphorylase functions only on α-1,4-glucosyl linkages on the terminal chains of glycogen; but the glycogen molecule is more complex in the types of bonds that bind the glucose units together. Glycogen is a highly branched homopolysaccharide containing only D-glucosyl units joined in α-1,4-glucosyl and in α-1,6-glucosyl linkages, probably arranged in a multiply-branched or "tree" structure, according to Stetten and Stetten (41). Two other enzymes are intimately associated with the synthesis and degradation of glycogen and therefore, with the structural arrangement of this molecule. These are amylo-(1,4 $\rightarrow$ 1,6) transglucosidase (branching enzyme) and amylo-1,6-glucosidase (debranching enzyme). They either transfer glucose units from the α-1,4-glucosyl linkages to α-1,6-glucosyl linkages or remove glucose in the α-1,6-glucosyl linkages from the glycogen molecule, as the names imply.

When phosphorylase has removed as many α-1,4-glucosyl linkages as is enzymatically practical (the α-1,4-glucosyl linkages of the glycogen chains have approached within one maltose unit of the α-1,6-glucosyl linkage (41)) the remaining glycogen is referred to as a limit dextrin. Therefore, the presence of phosphorylase-a does not insure that the enzyme is actively degrading glycogen. One inference from this discussion is that the glyconeogenesis occurring in muscle during the "glycine effect", could result from a continual formation of a limit

dextrin, making phosphorylase-a essentially non-functional under such conditions. If this were the case during the "glycine effect", then the continual formation of a limit dextrin would result in a glycogen of a different chemical structure than that normally deposited.

The large number of variables adds to the difficulty of interpreting the data presented in this thesis. In twelve groups of animals (four diets and three different stress conditions) correlation or lack of it between both the muscle and liver glycogen levels, and the activities of glycogen synthetase and phosphorylase require consideration of the known facts and variables and the understanding that unknown variables are present.

SUMMARY

The data are discussed in relation to the established "glycine effect" and the "blocking" of this effect. The "glycine effect" is the increased glycogen storage after stress, and the increased glycogen synthesis during recovery after stress in rats, as a result of prefeeding a diet containing added glycine. "Blockage" of this "glycine effect" was obtained when the diets fed contained added creatine.

Two enzymes concerned with glycogen metabolism, glycogen synthetase and phosphorylase, were assayed in tissues of rats exhibiting the "glycine effect" and in others in which the "glycine effect" had been "blocked." Conclusions from these enzymatic determinations are as follows:

1. Total phosphorylase activity did not differ statistically regardless of the diet prefed or the stress imposed upon the animal.
2. Phosphorylase-a (active phosphorylase) activities were increased above the activities of the non-stress animals after one and one-half hours stress and also following three hours recovery after stress. One exception was noted under results. The changes in phosphorylase-a activities follow what would be expected in non-stress and stressed animals in view of the muscle glycogen levels at these times.
3. Feeding diets with additions of creatine, glycine, or creatine plus glycine resulted in a decreased glycogen synthetase activity in non-stress rats.
4. After three hour recovery following stress, glycogen synthetase activity was greater in rats prefed creatine-

control diets than in animals prefed creatine-glycine diets.

5. The differences in glycogen synthetase activities are discussed in light of the possibility of enzyme induction or repression.

6. The data on enzyme activities reported in this thesis do little to aid in explaining the underlying mechanisms of the "glycine effect" or of the "blocked" "glycine effect."

REFERENCES

1. Breckenridge, B. M., & Crawford, E. J. Glycogen anythesis from uridine diphosphate glucose in brain. J. Biol. Chem., 1960, 235, 3054-3057.
2. Given, M., & Knox, W. E. The specificity of tryptophan analogues as inducers, substrates, inhibitors, and stabilizers of liver tryptophan pyrrolase. J. Biol. Chem., 1960, 235, 1716-1718.
3. Cori, G. T. The effect of stimulation and recovery on the phosphorylase-a content of muscle. J. Biol. Chem., 1945, 158, 333-339.
4. Cori, C. F., Cori, G. T., & Green, A. A. Crystalline muscle phosphorylase. III. Kinetics. J. Biol. Chem., 1943, 151, 39-55.
5. Cori, G. T., & Green, A. A. Crystalline muscle phosphorylase. II. Prosthetic group. J. Biol. Chem., 1943, 151, 31-38.
6. Cunningham, L., Barnes, J. M., & Todd, W. R. Maintenance of carbohydrate stores before and after insulin administration in rats prefed diets containing added glycine. Arch. Biochem. Biophys., 1948, 16, 403-407.
7. DiPietro, D. L., & Weinhouse, S. Hepatic glucokinase in the fed, fasted, and alloxan-diabetic rat. J. Biol. Chem., 1950, 235, 2542-2545.
8. Duncan, D. B. A significance test for differences between ranked treatments in an analysis of variance. Virginia J. Sci., 1951, 2, 171-189.

9. Fiske, C. H. & Subbarow, Y. Phosphocreatine. J. Biol. Chem., 1929, 81, 629-680.

10. Gomori, G. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. Clin. Med., 1942, 27, 955-958.

11. Good, C. A., Kramer, H., & Somogyi, M. The determination of glycogen. J. Biol. Chem., 1933, 100, 485-491.

12. Green, A. A. & Cori, G. T. Crystalline muscle phosphorylase. I. Preparation, properties and molecular weight. J. Biol. Chem., 1943, 151, 21-29.

13. Guest, M. M. Carbohydrate storage and metabolism in the rat. J. Nutrition, 1941, 22, 205-221.

14. Hauk, R. & Brown, D. H. Preparation and properties of UDPG-glycogen transferase from rabbitt muscle. Biochim. et Biophys. Acta, 1959, 33, 556-559.

15. Hauk, R., Illingworth, B., Brown, D. H., & Cori, C. F. Enzymes of glycogen synthesis in glycogen-deposition disease. Biochim. et Biophys. Acta, 1959, 33, 554-556.

16. Hess, W. C. & Shaffron, I. P. Rate of absorption and formation of liver glycogen by glycine. Proc. Soc. Exp. Biol. Med., 1952, 81, 404-405.

17. Illingworth, B. & Cori, G. T. Crystalline muscle phosphorylase. Biochem. Preparations, 1953, 3, 1-9.

18. Kachmar, J. F. & Boyer, P. D. Kinetic analysis of enzyme reactions. II. The potassium activition and calcium inhibition of pyruvic phosphoferase. J. Biol. Chem., 1953, 200, 669-682.

19. Keller, P.J. & Cori, C.F. Enzymic conversion of phosphorylase-a to phosphorylase-b. Biochim. et Biophys. Acta, 1953,12,235-238.

20. Kent, A.B., Krebs, E.G., & Fischer, E.H. Properties of crystalline phosphorylase-b. J. Biol. Chem., 1958,232,549-558.

21. Knox, W.E. Adaptive enzyme formation in animals. International symposium on enzyme chemistry, Tokyo and Kyoto, 1957, Vol.2, 414-416.

22. Krebs, E.G. & Fischer, E.H. Phosphorylase activity of skeletal muscle extracts. J. Biol. Chem., 1955,216,113-120.

23. Kvam, D.C. & Parks Jr.,R.E. Inhibition of hepatic-induced enzyme formation by 8-azaguanine. J. Biol. Chem., 1960,235,2893-2896.

24. Larner, J. & Villar-Palasi, C. A uridine coenzyme-linked pathway of glycogen synthesis in muscle. Biochim. et Biophys.Acta,1958,30,449.

25. Larner, J. & Villar-Palasi,C. Enzymes in a glycogen storage myopathy. Proc.Natl.Acad.Sci. U.S., 1959,45,1234-1235.

26. Larner, J.,Villar-Palasi,C., & Richman, D.J. Insulin stimulated glycogen formation in rat diaphragm. Ann.N.Y.Acad.Sci., 1959, 82,345-353.

27. Leloir, L.F. & Cardini, C.E. Biosynthesis of glycogen from uridine diphosphate glucose. J. Am. Chem. Soc., 1957,79,6340.

28. Leloir, L.F. & Goldemberg, S.H. Synthesis of glycogen from uridine diphosphate glucose in liver. J. Biol. Chem., 1960, 235,919-923.

29. Leloir, L.F., Olavarria, J.M., Goldemberg, S.H., & Carminatti, H. Biosynthesis of glycogen from uridine diphosphate glucose. Arch. Biochem. Biophys., 1959, 81, 508-520.

30. Lu. G. D. Studies on the metabolism of pyruvic acid in normal and vitamin B_1-deficient states. I. A rapid, specific, and sensitive method for the estimation of blood pyruvate. Biochem. J., 1939, 33, 249-254.

31. Madsen, N. B. & Cori, C. F. The binding of adenylic acid by muscle phosphorylase. J. Biol. Chem., 1957, 224, 899-908.

32. Mirski, A., Rosenbaum, I., Stein, L., & Wertheimer, E. On the behavior of glycogen after diets rich in protein and carbohydrate. J. Physiol., 1938, 92, 43-61.

33. Mommaerts, W. F. H. M., Illingworth, B., Pearson, C. M., Guillory, R. J., & Seraydarian, K. A functional disorder of muscle associated with the absence of phosphorylase. Proc. Natl. Acad. Sci. U. S., 1959, 45, 791-797.

34. Newburger, R. A. & Brown, F. R. The effect of ether and starvation on liver glycogen maintenance after various diets. Am. J. Physiol., 1942, 136, 746-749.

35. Olsen, N. S., Hemingway, A., & Nier, A. O. The metabolism of glycine. I. Studies with the stable isotope of carbon. J. Biol. Chem., 1943, 148, 611-618.

36. Ringer, A. J. & Lusk, G. The origin of dextrose from amino acids in phlorhizin glucosuria. Z. Physiol. Chem., 1910, 66, 106-119.

37. Robbins, P. W., Traut, R. R., & Lipmann, F. Glycogen synthesis from glucose, glucose-6-phosphate, and uridine diphosphate glucose in muscle preparations. Proc. Natl. Acad. Sci. U. S., 1959, 45, 6-12.

38. Ruisseau, J. P. du, Greenstein, J. P., Winitz, M., & Birnbaum, S. M. Studies on the metabolism of amino acids and related compounds *in vivo*. VI. Free amino acid levels in the tissues of rats protected against ammonia toxicity. Arch. Biochem. Biophys., 1957, 68, 161-171.

39. Schmid, R., Robbins, P. W., & Traut, R. R. Glycogen synthesis in muscle lacking phosphorylase. Proc. Natl. Acad. Sci., U. S., 1959, 45, 1236-1240.

40. Shaffer, P. A. & Somogyi, M. Copper-Iodometric reagents for sugar determination. J. Biol. Chem., 1933, 100, 695-713.

41. Stetten Jr., D. & Stetten, M. R. Glycogen metabolism. Physiol. Revs., 1960, 40, 505-537.

42. Talman, E. L. The relation of dietary glycine to glycogenesis in the rat. Thesis presented to the biochemistry department: University of Oregon Medical School, 1951.

43. Todd, W. R. & Allen, M. Synthesis of tissue glycogen in rats prefed diets with added glycine. Am. J. Physiol., 1958, 194, 338-340.

44. Todd, W. R. & Allen, M. Tissue glycogen synthesis in adrenalectomized rats fed glycine-containing diets and given hydrocortisone. Am. J. Physiol., 1958, 195, 643-644.

45. Todd, W. R., Barnes, J. M., & Cunningham, L. Maintenance of liver glycogen by rats fasted after feeding individual amino acids. Arch. Biochem. Biophys., 1947, 13, 261-264.

46. Todd, W. R. & Talman, E. On the glycuneogenetic action of fed glycine in the rat. Arch. Biochem. Biophys., 1949, 22, 385-392.

APPENDIX

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate (Adenylic Acid)
ATP	Adenosine triphosphate
EDTA	Ethylenediamine tetraacetic acid
G-1-P	Glucose-1-phosphate
G-6-P	Glucose-6-phosphate
Pi	Inorganic phosphate
UDP	Uridine diphosphate
UDPG	Uridine diphosphoglucose
UTP	Uridine triphosphate