DIETARY AND STRESS EFFECTS ON NADH-CYTOCHROME C REDUCTASE AND NITROGEN OF RAT HEPATIC MITOCHONDRIA

by

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I N T R O D U C T I O N

In 1938 Mirski and co-workers (18) compared the liver glycogen levels of rats fed high protein diets with those of rats fed high carbohydrate diets. These workers found that the liver glycogen levels of the animals on the high protein diet were lower. After 24 hours fast, however, the animals prefed the high protein diet had higher liver glycogen reserves than the rats prefed the high carbohydrate diet. They postulated increased glyconeogenesis to be the cause of this higher liver glycogen level and called this the "protein effect." This effect could not be demonstrated in adrenalectomized animals (18).

Todd and co-workers (28) demonstrated a similar maintenance of elevated liver glycogen levels in 24 hour fasted rats prefed a semisynthetic diet containing 10 percent glycine. This effect of glycine-feeding on carbohydrate stores was also demonstrated following a cold water swim-stress(30). Rats prefed the glycine containing diet maintained liver glycogen and blood glucose at higher levels after the swim stress than did animals prefed isocalorically on diets without added glycine. Glycine-fed animals permitted to recover from the swim-stress for three hours synthesized much liver glycogen while rats prefed the isocaloric control diet did not. This increased carbohydrate maintenance during stress and resynthesis during recovery in the animals fed the glycine containing diets constitutes the "glycine effect" (30).

The "glycine effect" could not be demonstrated in adrenalectomized animals. However, following the administration of cortisol to adrenalectomized animals the glycine effect was similar to the effect demonstrated in intact animals (31).

There is considerable evidence that glycine-feeding stimulates glycogen formation from other sources. The feeding of glycine-1-C^{13} to mice (19) resulted in increased liver glycogen 16 hours after feeding, but only a small portion of the C^{13} was incorporated into the glycogen.

Todd and co-workers (4) later showed that liver and muscle glycogen levels as well as blood sugar concentrations were considerably higher in glycine-fed, insulin treated animals than in control-fed insulin treated animals following an 8 hour fast and a 5 hour period of insulin action. These higher carbohydrate concentrations in the glycine-fed animals were not the result of direct conversion of stored glycine into carbohydrate (29). Had the extra glycine, found in the glycine-fed animals after an 8 hour fast, been directly converted into carbohydrate during the 5 hours insulin action, this direct conversion could have accounted for only about 1/6 of the excess carbohydrate in the glycine-fed animals (over the amount found in control-fed animals) (29).

Todd and co-workers (32) also found that animals prefed the glycine diet containing one percent added creatine not only lost the ability to demonstrate the glycine effect, but also had considerably decreased liver glycogen levels before

the swim-stress (Fig. 1)*. The latter liver glycogen concentrations were less than half the values found in the glycine-fed rats. During swim-stress the glycine-creatine-fed animals lost some liver glycogen, but the greatest difference between the liver glycogen concentration of the glycine-creatine-fed and the glycine-fed rats was evident following the three hour recovery period after the swim-stress. The glycine-creatine-fed recovered animals had liver glycogen levels averaging 0.39 percent wet weight (32) while the glycine-fed animals resynthesized liver glycogen increasing the concentration from 1.7 percent following swim-stress to 2.95 percent following recovery (30).

From the above observations it is apparent that ingested creatine seriously interferes with liver glycogen synthesis as well as with its maintenance.

Another facet of carbohydrate metabolism involves mitochondrial electron transport. That mitochondria are concerned with oxidation was suspected by early investigators (1). Clear evidence of the respiratory function of mitochondria, however, had to await the development of cell fractionation methods. Utilizing these methods for large scale preparations, mitochondria can now be isolated relatively uncontaminated by other cell fractions (10).

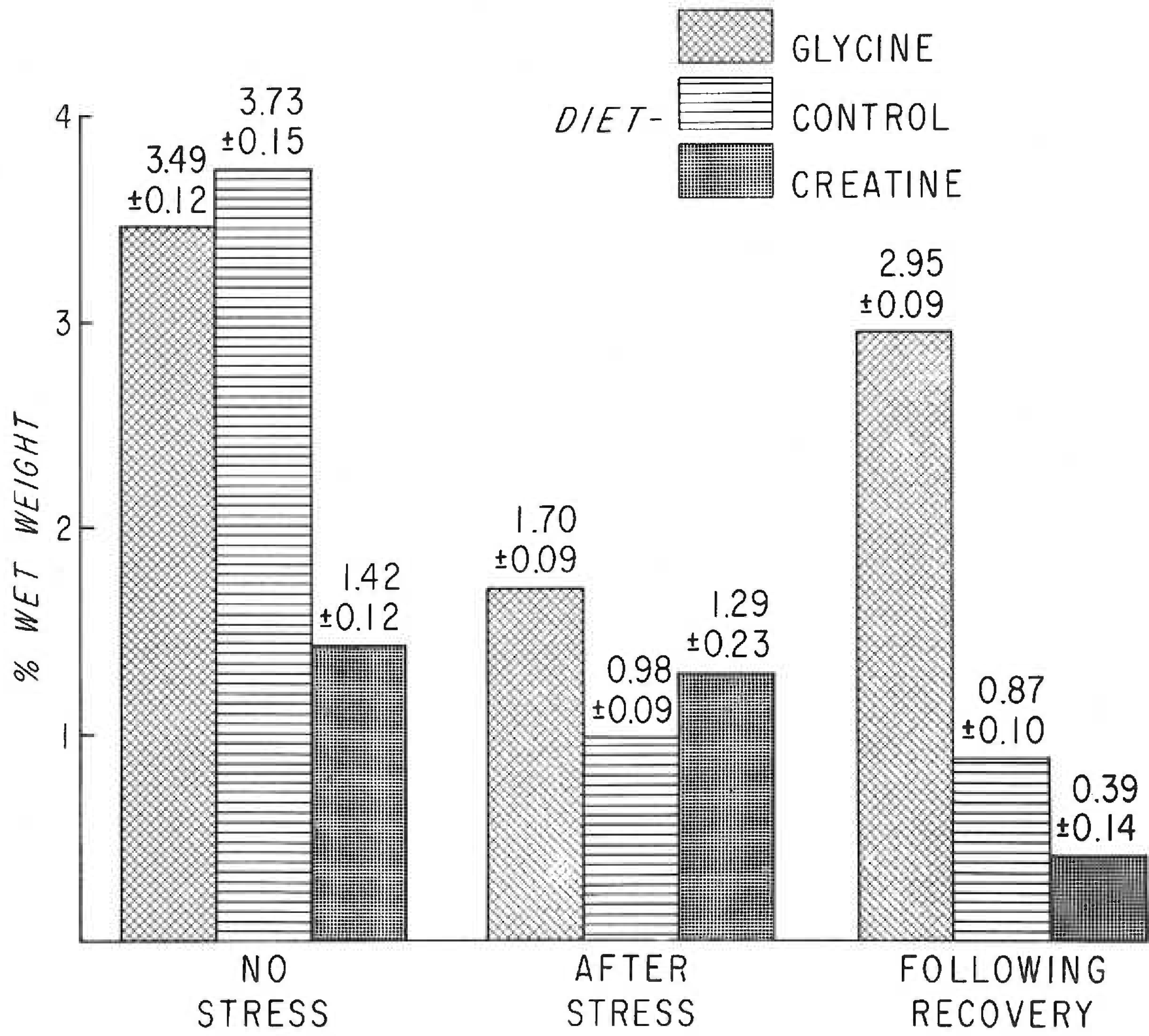
The oxidative properties of mitochondria are modified to some extent during the various isolation procedures.

*reproduced by permission of Dr. W. R. Todd (see bibliography 32.)

Figure 1

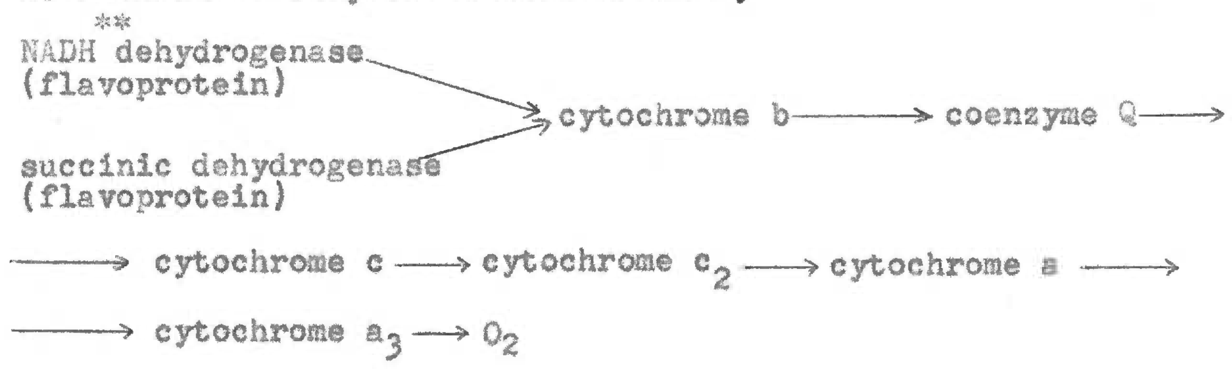
Rat liver glycogen levels, percent wet weight in rats prefed control, glycine or glycine-creatine diets and subjected to no stress, swim stress or recovery from stress. Reproduced by permission of Dr. W. R. Todd.

Todd, W.R., Harris, M., & Laastuen, L. Effects of creatine feeding on liver glycogen and liver enzymes concerned with glucose-6-phosphate metabolism. Pacific Slope Biochemical Conference, September 1962, Seattle, Washington (32).



GLYCINE
DIET-
CONTROL
CREATINE
% WET WEIGHT
4
3
2
1
3.49
±0.12
3.73
±0.15
1.42
±0.12
1.70
±0.09
0.98
±0.09
1.29
±0.23
2.95
±0.09
0.87
±0.10
0.39
±0.14
NO
STRESS
AFTER
STRESS
FOLLOWING
RECOVERY

If the mitochondrial electron transport system is represented as a chain of enzymes in this manner*,



it is evident that loss of a member of this chain will disrupt the electron transport system. Cytochrome c requirements of NADH dehydrogenases from different mitochondrial preparations vary. Mackler and Green (17) suggested that in some preparations cytochrome c is lost, due to its solubility.

Another possible explanation of the confusion regarding NADH dehydrogenating enzymes is offered by King and Howard (15). These investigators proposed that the original site of reaction on the enzyme loses access to the next member due to the rupture of the respiratory chain. New sites for electron transport are uncovered by the action of the reagents used in the preparation of the material. Cytochrome c may thus become a nonphysiological electron acceptor.

A recent theory described by Ringler and co-workers (22) accounts for the differences in cytochrome c requirement of dehydrogenases prepared by different extraction procedures in still another way. They suggested that the enzyme may

*The exact makeup of such an electron transport system is not agreed upon by various workers in the field.

**NADH - new name for reduced diphosphopyridine nucleotide (DPNH)

undergo structural alteration, such as a partial breakdown or fragmentation during isolation. Such structural alteration was thought by Watari and co-workers (33) to transform the original NADH dehydrogenase into NADH-cytochrome c reductase in heart mitochondria.

In any event, NADH is dehydrogenated by deoxycholate treated mitochondria upon the addition of cytochrome c.

Mitochondrial preparations with NADH oxidase activity (NADH oxidation by molecular oxygen) have also been found to develop cytochrome c reductase activity upon the addition of deoxycholate (3).

Another major function of mitochondria besides the mediation of electron transport and its coupling to phosphorylation is the catalysis of the citric acid cycle oxidations. The transformation of the chemical energy, formed through these oxidations into bond energy of high energy phosphates in the mitochondria earned these structures the designation "biological transducers" (11). Since glucose is a precursor of pyruvate and of acetyl CoA, this sugar is indirectly a major substrate of these mitochondrial enzymes. Electrons from glucose oxidation funnel into the mitochondrial electron transport chain through NADH. Thus changes in carbohydrate metabolism may be reflected in the rate of mitochondrial NADH dehydrogenation.

From the foregoing several significant problems are posed. One of these involves the relation of the abolition

of the "glycine effect" by creatine ingestion to the NADH dehydrogenating capacity of liver mitochondria. Studies have been done in this relation in mitochondrial preparations from livers of animals fed the various diets and from such animals sacrificed before stress, immediately after stress, and following recovery from stress. The assays involved the determination of NADH-cytochrome c reductase activity in deoxycholate treated preparations containing added cytochrome c.

Nitrogen determinations were done on all mitochondrial preparations studied since it is desirable to express activity of the enzyme on the basis of specific activity per mg of nitrogen.

E X P E R I M E N T A L

DIETS

Table 1 presents the composition of the semisynthetic diets used. In the glycine diet 10 percent of the dextrin was replaced by an equal weight of glycine. In the glycine-creatine diet in addition to the 10 percent glycine, 1 percent creatine was included.

ANIMALS

Male Sprague-Dawley strain rats weighing from 150 to 200 grams were used. The animals were removed from a Purina Laboratory Chow stock diet and placed on the control diet for 24 hours to help accustom them to the synthetic rations. Part of the group was maintained on the control ration and others were given the appropriate experimental diet for 20 hours. At the end of this period, the last meal was given after dividing it into two parts. Part a) contained all the constituents of the control diet except dextrin. Two grams of this diet were administered at 2 A.M. Part b) of the diet contained glucose instead of dextrin to facilitate administration. In addition to glucose part b) contained glycine, glycine and creatine, or no addition depending on which regimen the animals were on during the previous 20 hours. One g of part b) was administered by gastric gavage in 2 mls water 2 hours after part a) has been given. At this time part a) had practically always been consumed. Total food consumption during the 48 hours that the animals were on the synthetic diet was 40 g; 20 g control ration during the first

TABLE 1

COMPOSITION OF DIETS

in percent by weight

	Control ration	Glycine ration	Glycine-creatine ration
Casein	16	16	16
Brewer's yeast[1]	10	10	10
Salt mixture[2]	10	10	10
Cod liver oil	2	2	2
White corn dextrin	54	44	44
Glucose	8	8	8
Glycine	0	10	10
Creatine	0	0	1

1 Squibb

2 Nutritional Biochemicals Corporation (salt mix W)

24 hours, 17 g of the appropriate ration during the following 20 hours, 2 g a) diet, and finally 1 g b) diet. The administration of the diet in the described manner resulted in close control of total food intake and in control of the time of food intake prior to the short (4 hours) fast preceding the stress period.

Some of the animals were sacrificed without stress 4 hours after stomach tubing. These rats will be referred to as non-stressed. Other animals were made to swim in 14°C water for 10 minutes, then "rested" at room temperature (25°C) for 30 minutes. The swim and "rest" were repeated. Finally the rats were forced to swim an additional ten minutes. During the two "rest" periods the rats were wet and shivering, so that the total stress amounted to 90 minutes.

Immediately following the last swim period some of the animals were sacrificed. These will be referred to as the stress animals. Other rats were allowed to recover at 25°C for 3 hours before they were sacrificed. They are referred to as the recovery animals. All animals were anaesthesized by intraperitoneal Nembutal injection (4 mg / 100 g body weight).

HANDLING OF TISSUES AND METHODS

The liver was removed, blotted, weighed and minced in a garlic press. Approximately a one g sample of the mince was transferred into 2 ml of 30 percent KOH in a weighed centrifuge tube. This part of the procedure required about

60 seconds. The weight of the aliquot was recorded and the glycogen concentration of the sample was determined by the method of Good, Kramer and Somogyi (9). Glucose was estimated by the Shaffer-Hartman method, Somogyi modification (23) after 2½ hours hydrolysis of the glycogen in N H_2SO_4 at 100°C. In some instances a piece of tissue was removed for microscopic study before mincing. The tissue was cut with a razor blade and briefly washed in 0.25 M sucrose. It was then placed in Fleming's fixative. Later it was imbedded in paraffin, sectioned and stained with iron hematoxylin.

The rest of the liver mince was immediately chilled and handled from here on at 0° to 4°C. A suitable portion (2 to 4 g) of the mince was weighed for the preparation of the mitochondrial fraction. This aliquot of the mince was homogenized in two volumes of 0.25 M sucrose in a Potter-Elvehjem homogenizer. It was quantitatively transferred to 50 ml centrifuge tubes and diluted 1:4 (liver weight/volume) with 0.25 M sucrose. The mitochondrial fraction was prepared by the Fairhurst, Maher and Smith modification (5) of the Schneider and Hogeboom method (24).

The homogenate was centrifuged at 600 x g for 5 minutes in an International model PR2 centrifuge at 4°C. The sediment consisting of blood cells and intact liver cells was discarded. The supernate was centrifuged at 24,000 x g for 20 minutes. The resulting pellet was twice resuspended, washed and recentrifuged in 0.25 M sucrose. The final pellet was frozen.

Microsomal contamination of different preparations was compared by the assay of glucose -6- phosphatase activities. This enzyme is localized predominantly, if not exclusively, in the microsomal fraction (25). Glucose-6-phosphate was added in suitable concentration to the diluted preparation at pH 6.8 according to the method of Freedland and Harper (6). The liberated inorganic phosphate was determined by the method of Taussky and Shorr (27).

The water content of the liver was determined by drying weighed aliquots of the mince at 100°C for 48 hours and reweighing.

NADH-cytochrome c reductase activity was assayed by following the disappearance of added NADH from the assay mixture at 340 mμ wavelength (16) in a Beckman model DU spectrophotometer. The mitochondrial preparations used for the assay were resuspended by hand - homogenizing in 0.25 M sucrose in the cold. The homogenate was diluted with 1 ml cold 0.25 M sucrose per gram of original liver. Part of this suspension was further diluted 1:6 with 0.25 M sucrose and a sufficient amount of deoxycholate to give a final deoxycholate concentration of 0.067 percent. An aliquot of the latter mixture, usually 0.05 ml, was blown into the cuvette containing all other constituents of the assay mixture to give a total volume of 3 ml. The assay mixture had the following composition:

phosphate buffer pH 7.6	5 mM
NADH (disodium salt)	68 μM
cytochrome c	17 μM

The temperature was maintained at 37°C during the assay.

It was established that neither the substrate (NADH), nor the cytochrome c concentrations were rate limiting and that NADH was not autoxidized in the reaction mixture in the absence of the mitochondrial preparation. It was also established that the procedure as employed gave optical density changes which were linear with time.

Enzyme activity was expressed as µmoles substrate (NADH) dehydrogenated per minute per mg mitochondrial nitrogen at 37°C. Calculations were based on the A_m (molar absorbancy) of NADH being equal to 6.22×10^3 as established by Horecker and Kornberg (13).

The influence of glycine, creatine and phosphocreatine on the enzyme activity in vitro was studied by adding these substances to the preparations in suitable concentrations.

Nitrogen concentrations of the samples were determined by the micro Kjeldahl method.

The non-protein nitrogen contents of several mitochondrial preparations were also determined by dissolving the preparation with an excess of sodium bicarbonate, and precipitating the protein by the addition of perchloric acid. The nitrogen content of the filtrate (NPN) was then determined by the micro Kjeldahl method.

Statistical analysis of the various types of data presented was done by the Student *t* test unless otherwise indicated.

R E S U L T S

Table 2 presents NADH-cytochrome c reductase activities of hepatic mitochondria from rats prefed the three different rations; control diet, glycine diet, and glycine-creatine diet--under the three different physiological conditions studied (as described in the methods section). Each value is the average result from 6 to 8 animals with the standard error of the mean. The enzyme activity is expressed as specific activity, i.e., µmoles NADH oxidized per minute, per mg mitochondrial nitrogen.

Significant changes in enzyme activity were found under two conditions. Specific activity of NADH-cytochrome c reductase was increased in stressed glycine-fed rats compared to nonstressed and also to recovery animals on this diet. The increased activity of the enzyme was due to the combined effects of glycine feeding and stress. The other significant change in enzyme activity was found in glycine-creatine-fed nonstressed animals compared to glycine-fed non-stressed rats.

In order to study possible _in vitro_ activating or inhibiting effects of glycine or creatine on the NADH-cytochrome c reductase activity, samples from control-fed non-stressed animals were assayed for the enzyme activity with and without addition of glycine, creatine or creatine phosphate. Results of five such enzyme assays are presented in Table 3. Statistical evaluation of these results by the analysis of variance method indicates that there was no _in vitro_

TABLE 2

NADH-cytochrome c reductase activity of liver mitochondria in rats prefed control, glycine, or glycine-creatine diets and subjected to no stress, swim-stress, or recovery from stress.

Specific activity NADH-cytochrome c reductase per mg of mitochondrial nitrogen			
Diet	No stress	Stress	Recovery from stress
Control	2.30±0.17	2.47±0.21	2.30±0.25
Glycine	2.29±0.07	2.75±0.11*	2.23±0.60
Glycine-creatine	1.92±0.11**	2.66±0.27	1.98±0.20

Each value is the average of 6 to 8 animals and is expressed as specific activity/mg mitochondrial nitrogen ± standard error

Specific activity = μmoles NADH dehydrogenated/minute

*Statistically different as compared to glycine-fed nonstressed and glycine-fed recovery values, p= 0.01

**Statistically different as compared to glycine-fed non-stressed values, p = 0.01

TABLE 3

The effect of *in vitro* additives: glycine, creatine, or creatine phosphate on the NADH-cytochrome c reductase activities of hepatic mitochondria from control-fed non-stressed rats.

Specific activity NADH-cytochrome c reductase per mg of mitochondrial nitrogen					
Additive/g liver (wet weight)					
Without additive	μg Glycine 25	μg Glycine 200	μg Creatine 25	μg Creatine 200	mM Creatine phosphate 4.6
1.31	1.38	1.35	1.37	1.35	1.35
1.33	1.27	1.29	1.29	1.32	1.27
1.43	1.32	1.39	1.42	1.26	1.38
1.85	1.84	2.07	1.82	1.92	1.93
1.82	1.71	1.72	1.75	1.71	1.90

Statistical evaluation by analysis of variance between groups (F test)

Variances are equal, $p = 0.05$

inhibition or activation of NADH-cytochrome c reductase activity due to the addition of glycine or creatine to the assay mixture, either at physiological concentrations (25μg/g liver wet weight), or in large excess (200 μg/g liver wet weight). Likewise the addition of creatine phosphate in large excess had no effect on the enzyme activity. The possibility of activation by glycine and stress *in vivo*, however, cannot be excluded.

The nitrogen contents of mitochondrial preparations are presented in Table 4. Each value is the average of results from 6 to 8 animals with the standard error of the mean. Several significant differences in these values are as follows:

a) Mitochondrial preparations from control-fed recovery animals showed increased nitrogen concentrations compared to stressed animals on the same diet.

b) Glycine-creatine-fed recovery values were elevated over those from glycine-creatine-fed stressed animals.

c) Glycine-fed animals, in contrast to rats on the two other diets, did not show increased nitrogen after recovery.

d) Glycine-creatine-fed non-stressed animals showed increased nitrogen compared to non-stressed animals on each of the other diets.

e) Glycine-creatine-fed recovery animals had increased mitochondrial nitrogen compared to recovery animals on both the other diets.

TABLE 4

Hepatic mitochondrial nitrogen concentrations in rats prefed control, glycine, or glycine-creatine diets and subjected to no stress, swim-stress or recovery from stress.

Mg hepatic mitochondrial nitrogen/g liver(wet wt.)			
Diet	No stress	Stress	Recovery from stress
Control	4.06±0.49	5.02±0.62	5.69±0.63[a]
Glycine	4.05±0.38	5.57±0.81	5.56±0.60
Glycine-creatine	5.43±0.51[b]	6.36±0.38	8.91±0.43[a,b,c]

Each value is the average of 6 to 8 animals ± standard error

a Statistically different as compared to stress values, same diet, $p = 0.01$

b Statistically different as compared to the other two diets, same physiological condition, $p = 0.01$

c Statistically different as compared to non-stress values, same diet $P = 0.01$

f) The nitrogen concentration of the preparations from glycine-creatine-fed recovery animals was greatly increased compared to the values in the non-stressed and stressed conditions of animals prefed this diet.

These changes in nitrogen concentration were not due to differences in liver hydration. The water contents are presented in Table 5. Each value represents the average of 6 animals with the standard error of the mean. The water contents are expressed as percent of wet weight and no significant differences were found.

Non-protein nitrogen was determined in hepatic mitochondrial preparations from 4 animals each under the following conditions: control-fed non-stressed, glycine-creatine-fed non-stressed, and glycine-creatine-fed recovery. From the data in Table 6 it is apparent that there were no differences in the ratio of non-protein nitrogen to the total mitochondrial nitrogen.

In general, mitochondrial preparations contain microsomes. The degree of this contamination may be roughly estimated by finding the activity of glucose-6-phosphatase in the preparations, since this enzyme is predominantly, if not exclusively, localized in the microsomes, in the form of a tightly bound membrane-enzyme (25). Assay of the activity of this enzyme was undertaken in order to find out how much the higher centrifugal force used for the sedimentation of the mitochondrial fractions (see methods) increased

TABLE 5

Water content of liver from rats fed control, glycine, or glycine-creatine diet and subjected to no stress, swim-stress or recovery from stress.

% water wet weight			
Diet	No stress	Stress	Recovery from stress
Control	71.11±2.47	71.73±3.16	71.98±1.52
Glycine	71.02±2.34	69.91±1.65	71.44±0.85
Glycine-creatine	70.93±2.28	68.74±2.00	70.61±1.55

Each value is the average of 6 animals with standard error of the mean

None of the differences in water content are statistically significant, $p = 0.05$

TABLE 6

Non-protein nitrogen concentrations of mitochondrial preparations from rats prefed control or glycine-creatine diets and subjected to no stress or recovery from stress.

Non-protein nitrogen as % of total nitrogen		
Diet	No stress	Recovery from stress
Control	9.1±0.3	-
Glycine-creatine	10.1±0.6	9.9±0.5

Each value is the average of 4 animals ± standard error

No statistically significant differences, p = 0.05

microsomal contamination beyond that of the preparations fractionated by the original method of Schneider and Hogeboom (24).

Table 7 presents the results of glucose-6-phosphatase and NADH-cytochrome c reductase assays together with the nitrogen concentrations of mitochondrial preparations from colony rats maintained on Purina Laboratory Chow. The preparations differ only in the speed of centrifugation. The values are the average of determinations on 6 different animals, with standard error of the mean. Glucose-6-phosphatase activity is expressed as µmoles of phosphorus split from glucose-6-phosphate per minute per mg nitrogen at 30°C.

On the basis of the glucose-6-phosphatase findings, microsomal contamination of the two preparations did not differ. Further evidence for this is found in the unchanged NADH-cytochrome c reductase activities of these preparations. This activity would have increased in case of increased microsomal contamination, since almost twice as much activity is reported for this enzyme in the microsomal fraction compared to the mitochondrial fraction (12), (2).

Since a tremendous increase in the nitrogen content of the mitochondrial fraction was found in glycine-creatine-fed animals, especially following recovery from stress, glucose-6-phosphatase activities of some of these preparations were also assayed, to see if the increase in nitrogen could be of microsomal origin. Table 8 shows that microsomal

TABLE 7

The effect of two different centrifugal forces on glucose-6-phosphatase activity, NADH-cytochrome c reductase activity and nitrogen content of mitochondrial preparations from rats maintained on Purina Laboratory Chow.

Centrifugal force	Specific activity/mg mitochondrial nitrogen		mg nitrogen/ g liver(wet wt.)
	Glucose-6-phosphatase	NADH-cytochrome c reductase	
6000 X g	0.38±0.02	3.50±0.14	4.06±0.39
24000 X g	0.44±0.02	3.17±0.16	4.69±0.31

Specific activity = μmoles substrate utilized/minute

Each value is the average of 6 animals ± standard error

None of the differences are statistically significant, $p = 0.05$

TABLE 8

Glucose-6-phosphatase activity of hepatic mitochondrial preparations from rats prefed control, or glycine-creatine diets and subjected to no stress, or recovery from stress.

Glucose-6-phosphatase, Specific activity/mg nitrogen		
Diet	No stress	Recovery from stress
Control	0.57±0.02	-
Glycine-creatine	0.74±0.06	0.92±0.02*

Each value is the average of 4 or 5 animals

Specific activity /mg mitochondrial nitrogen ± Standard Error

Specific activity = μmoles inorganic phosphate liberated per minute at 30°C

*Significant difference between control no stress and glycine-creatine-recovery, $p = 0.01$

contamination indeed increased in the glycine-creatine-fed recovery animals.

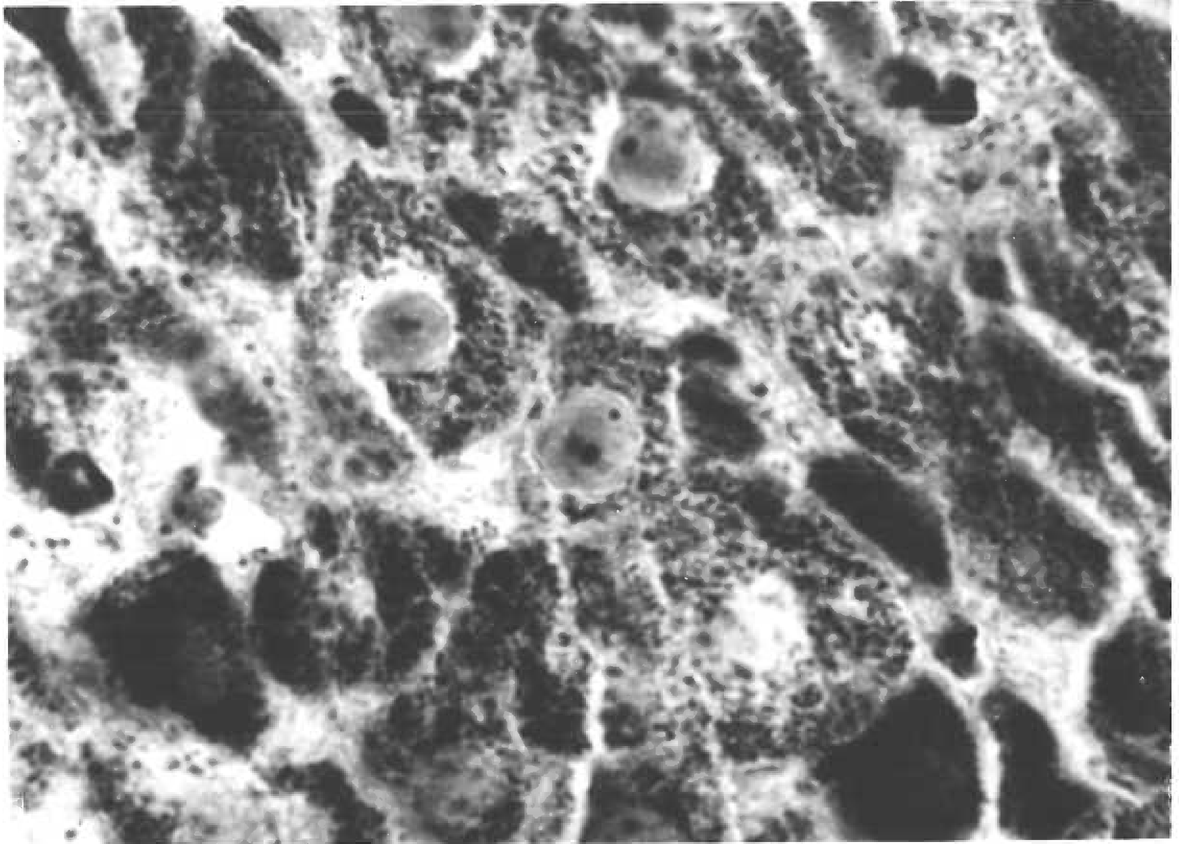
Photomicrographs of hematoxylin stained liver sections from glycine-fed recovery and glycine-creatine-fed recovery animals are found in Figure 2. These show differences in the size and possibly in the shape of hepatic mitochondria.

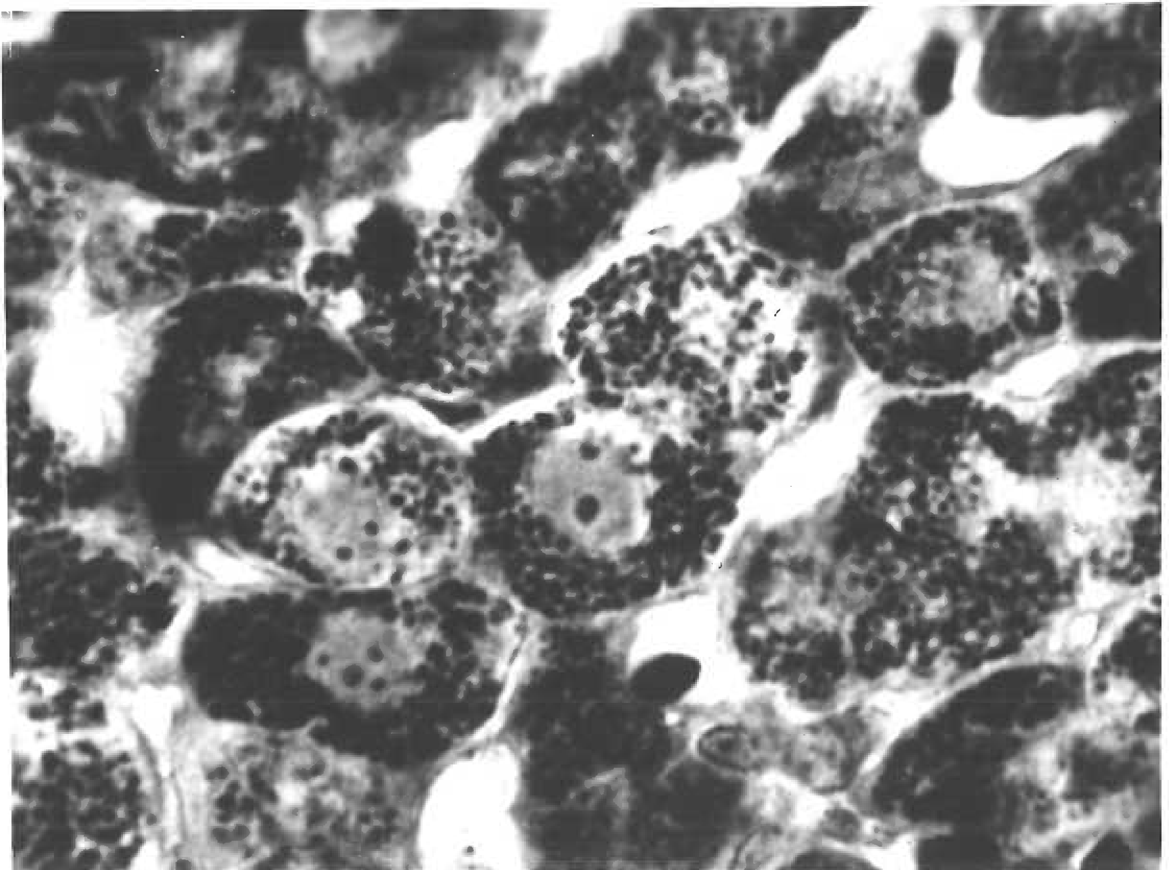
Figure 2

Photomicrograph of hematoxylin-stained liver section, x 1500.

a) glycine-fed recovery after stress

b) glycine-creatine-fed recovery after stress





D I S C U S S I O N

Stripe and Schwartz (26) reported decreased NADH-cytochrome c reductase activity in the liver mitochondria of rats fed a protein deficient diet for 13 days. The enzyme activity in this case was restored upon the addition of a full complement of amino acids to the diet, although this required 3 to 6 days.

The increased enzyme activity reported herein immediately after stress in the glycine-fed animals also constitutes a diet induced change. Although addition of glycine in the *in vitro* enzyme assay does not influence the enzyme activity, the effects of glycine coupled with stress *in vivo* could be due to enzyme activation.

The means of differentiation between enzyme activation and adaptation are indirect. For example Pogell (21) found that rabbit liver fructose-di-phosphatase activities were increased after fructose feeding, but at the same time the *in vitro* activation of this enzyme by proteolysis was less than the activation of the enzyme from control-fed rabbit tissue. This difference in *in vitro* activation was explained on the basis of a pre-existing *in vivo* activation of the enzyme in the fructose-fed rabbit. Friedland and Harper (7) assaying the activity of fructose-di-phosphatase from rat liver under similar conditions of glucogenic stress, also found the enzyme activity increased. However, *in vitro* activation of fructose-di-phosphatase from the stressed rats

was not greater than similar activation of the enzyme from control rats. These findings suggested adaptive enzyme formation. Further evidence for adaptive enzyme formation was found by these workers (8) and also by Kvam (14) when, upon ethionine administration to the animals enzyme synthesis was inhibited. This inhibition was relieved by methionine administration.

In the present work net synthesis of enzyme protein, differentiated from other proteins in the mitochondrial fraction, could be studied by similar methods. If ethionine administration to glycine-fed rats prevents the increase of hepatic NADH-cytochrome c reductase activity, this would be presumptive evidence for adaptive enzyme formation. This avenue of approach has not been explored.

Whatever the mechanism of the increase in hepatic NADH-cytochrome c reductase activity (Table 2), it seems to be another manifestation of the "glycine effect" since it occurs in the glycine-fed stressed animal and it is in these rats that high liver glycogen levels are maintained.

The nitrogen content of the mitochondrial preparations was increased following recovery (compared to after stress) in the control-fed and the glycine-creatine-fed animals. In glycine-fed animals the absence of the increase in nitrogen may be another manifestation of the "glycine effect", since this difference in metabolism (i.e., no increase in nitrogen content) occurred in those animals, which, due to glycine

feeding, synthesized and redeposited large amounts of liver glycogen upon recovery from the swim-stress.

The differences found in mitochondrial nitrogen led to a study of the non-protein nitrogen of these preparations (Table 6). For this determination the experimental conditions were chosen in which the increase in nitrogen was greatest. Non-protein nitrogen contents of such preparations were compared with preparations containing the least total nitrogen. That non-protein nitrogen remained proportional (at about 10 percent of the total nitrogen) indicates that the bulk of the additional nitrogen, in these mitochondrial preparations, is of protein origin. This increase in nitrogen may be the result of protein synthesis or redistribution. The possibility exists that creatine feeding exerts some kind of directive influence on the metabolism of this cell fraction. A possible result of such influence could be that energy available from the effects of glycine-feeding, instead of being utilized for glycogen synthesis and deposition in the liver, is used for other purposes, among them protein synthesis or redistribution. In this way the inclusion of creatine in the glycine containing diet could abolish the "glycine effect".

It is surprising that during the short duration of the swim-stress and recovery (4.5 hours) significant changes in protein synthesis or mobilization could take place. However, a recent report by Penn (20) recounts high rates of protein synthesis in liver mitochondrial cultures _in vitro_ in as

short a period of time as one hour.

A close negative correlation ($r = -0.83$) measured by the Pearson Product Moment correlation method*, has been found between the effects of glycine-creatine-feeding on a) liver glycogen deposition (Fig. 1) and on b) changes in mitochondrial nitrogen (Table 4). The largest increase in mitochondrial nitrogen, and the greatest deficiency in liver glycogen were found in the glycine-creatine-fed recovery rats. The glycine-creatine-fed stressed rats showed the least decrease in liver glycogen and the least increase in mitochondrial nitrogen of the rats in the three physiological conditions prefed this diet. The non-stressed animals showed changes in the above-mentioned variables which are of intermediate magnitude compared to the other two physiological conditions.

The correlation between the decreased hepatic glycogen maintenance and the increased mitochondrial nitrogen as an effect of creatine ingestion further increases the possibility of the directive influence of creatine discussed previously.

Another effect of the glycine-creatine-feeding was the decreased NADH-cytochrome c reductase activities found in the non-stressed rats (Table 2). This finding is difficult to interpret in view of its occurrence together with an increase in nitrogen content. The protein composition of the preparation could have changed. Attempts at separating the protein fractions of the mitochondrial preparations by

*correlation coefficient $r = \dfrac{N\Sigma xy - \Sigma x\Sigma y}{[N\Sigma x^2 - (\Sigma x)^2][N\Sigma y^2 - (\Sigma y)^2]}$

various techniques of zone electrophoresis were unsuccessful. Among the methods tried were paper, cellulose acetate and starch-gel electrophoresis with various buffers in the pH range 7.6 to 11.0.

Increased microsomal contamination of the mitochondrial fraction was apparent in glycine-creatine-fed recovered rats (Table 8). The constancy of the NADH-cytochrome c reductase activities (Table 2) in these preparations, however, contradict the findings in regards to increased microsomal contamination, since, as already mentioned, microsomes display a considerably higher NADH-cytochrome c reductase activity per mg protein than do mitochondria. Increased microsomal contamination, therefore, should result in an increased enzyme activity unless the protein composition of one or both fractions changed. Such a possibility cannot be discounted. These data again indicate the possibility of a directing force due to a combination of creatine ingestion and stress plus recovery.

Microscopic studies of stained liver sections reveal qualitative differences in the staining properties of mitochondria in hepatic parenchymal cells. The photomicrographs (1000 X magnifications of hematoxylin stained paraffin sections) reveal that the mitochondria are larger and stain darker in the glycine-creatine-fed rats following recovery compared to glycine-fed animals in the same physiological condition.

SUMMARY

Previous work has demonstrated that rats pre-fed a semisynthetic diet containing 10 percent glycine maintain liver glycogen and blood glucose following a swim-stress at higher levels than rats prefed the semisynthetic diet without added glycine. Glycine-prefed animals permitted to recover 3 hours after the swim-stress synthesize much liver glycogen, while control-fed animals do not. This increase in carbohydrate levels in the glycine-fed animals is referred to as the "glycine effect". The "glycine effect" is abolished in animals prefed the glycine diet containing one percent creatine.

In this thesis it is shown that the NADH-cytochrome c reductase activity was increased 20 percent in liver mitochondria of rats prefed the glycine diet and then stressed by cold water swimming. A small but significant decrease of this enzyme was found in rats prefed the same diet containing one percent creatine and not subjected to the stress. No changes in NADH-cytochrome c dehydrogenase activity were observed under the other conditions of diet or stress.

Mitochondrial nitrogen concentration was increased in the rats prefed the control-diet or the glycine-creatine-diet following recovery from the swim-stress. Glycine-prefed rats however, did not have increased mitochondrial nitrogen following recovery. These findings may be involved in the mechanism responsible for the "glycine effect".

Mitochondrial nitrogen concentrations were increased in the glycine-creatine-fed rats in inverse proportion to the decrease in liver glycogen in these animals. The correlation between these two variables and the likelihood that these nitrogen changes are due to changes in protein concentration give credence to the idea that creatine ingestion has a directive influence on some aspects of hepatic protein metabolism. Accordingly, as glycine ingestion (with stress) increases glycogen synthesis and deposition in the liver, additional creatine ingestion causes increased synthesis or mobilization of mitochondrial elements.

Microsomal contamination is judged to be increased in the preparations from glycine-creatine-fed animals, on the basis of increased glucose-6-phosphatase activities. NADH-cytochrome c reductase activities, however, did not increase on the basis of activity per mg nitrogen. Furthermore, this enzyme activity was decreased in the non-stressed rats prefed this diet. These findings give rise to the possibility of a changed protein composition in the mitochondrial, the microsomal, or both fractions.

Microscopic studies of hematoxylin stained liver sections attest to increases in the sizes of mitochondria in the parenchymal cells of glycine-creatine fed rats following recovery from swim stress compared to glycine-fed rats under the same physiological condition.

A C K N O W L E D G E M E N T S

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