RELATION OF AMINO ACID IMBALANCE TO CARBOHYDRATE STORAGE IN STRESSED RATS

by

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

THE GLYCINE EFFECT

Liver glycogen levels of rats fed various diets have been compared repeatedly with reference to the protein and carbohydrate contents of the diets. Thus, in 1938 Mirski and coworkers (46)found that upon 24 hours fast the liver glycogen levels of rats prefed a diet containing 70 percent casein were much higher than those of rats fed an isocaloric diet with 20 percent casein. This difference in liver glycogen could not be demonstrated in adrenalectomized rats. The effect of protein feeding on the liver glycogen stores was postulated to be due to increased gluconeogenesis.

Todd and coworkers (72) demonstrated a similar maintenance of liver glycogen levels in 24 hour fasted rats prefed a semisynthetic diet, adequate in protein and containing 10 percent glycine. This effect of glycine feeding was also demonstrated following a cold water swim stress (74). Glycine fed animals permitted to recover from the swim stress for three hours synthesized a considerable amount of liver glycogen, while rats prefed the isocaloric diet did not. Glycine fed rats also maintained higher liver glycogen levels immediately following swim stress than the control fed animals. The increased carbohydrate maintenance during stress and resynthesis during recovery in the glycine fed animals constitutes the "glycine

effect". This effect of glycine feeding could not be demonstrated in adrenalectomized animals (75). However, following the administration of equal quantities of cortisol to adrenalectomized glycine fed and control fed animals, the glycine effect was similar to the effect demonstrated in intact animals. Upon administration of a large excess of hydrocortisone, however, the carbohydrate reserves found in the glycine and control fed animals were equally high. Since the biosynthesis of the key enzymes of gluconeogenesis is known to be induced by adrenocortical hormones (82), these results merely indicate that excess adrenocorticoids are an overwhelming gluconeogenetic stimulus.

Todd and coworkers have shown (16) that liver and muscle glycogen levels as well as blood sugar concentrations were considerably higher in glycine fed insulin treated rats than in control fed insulin treated animals following an 8 hour fast and a 5 hour period of insulin action. The higher carbohydrate concentrations in the glycine fed animals were not the result of direct conversion of stored glycine into carbohydrate. 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 amounts found in control fed animals) (73).

There is also direct evidence that glycine feeding stimulates glycogen formation from other sources (50). Feeding of glycine-1-C^{13} to mice resulted in increased liver glycogen 16 hours after feeding, but only one carbon out of about 29 was labeled.

INFLUENCE OF THE GLYCINE EFFECT ON ENZYMES OF CARBOHYDRATE METABOLISM

Changes in hepatic enzyme activities have been reported during increased gluconeogenesis due to various stimuli, by a number of investigators. Some of the findings are contradictory. DiPietro and Weinhouse (17) found hepatic glucokinase activity decreased in fasting and also in diabetic rats. Vester (78) however, found no differences in liver glucokinase activity between fasted, diabetic, and fed normal rats. Glucokinase activity was also found to be insulin dependent, and not affected by adrenalectomy, nor by hypophysectomy (9).

Weber and coworkers (79, 80, 82) found that cortisone administration to adrenalectomized and to normal rats selectively increased the activities of hepatic gluconeogenetic enzymes. The enzymes that showed increased activities include glucose-6-phosphatase, phosphohexose isomerase, and fructose-1,6-diphosphatase. Glucose-6-phosphate dehydrogenase and phosphoglucomutase activities were not increased under the same conditions. The cortisone induced increases in enzyme activities were reported to be counteracted by ethionine and by actinomycin administration (81, 82). These increases in enzyme

activities are considered to involve de novo enzyme synthesis, rather than enzyme activation.

Weber and coworkers (79, 81) also found selectively increased enzyme activities in rats refed after long periods of fast. Fitch and Chaikoff (20) found similar changes in alloxan diabetic rats — another condition where carbohydrate formation is accelerated. Fitch and Chaikoff, among other investigators, believe that the levels of rate limiting enzyme activities are related to the metabolic activity of the path in which the enzymes participate. Weber (81, 82) suggests that the key enzymes glucose-6-phosphatase, fructose-1,6-diphosphatase, phosphoenolpyruvate carboxylase, and pyruvate carboxykinase, are not only the rate limiting enzymes along the gluconeogenetic pathway, but that their synchronous rise and fall may indicate that they occupy a single functional genome unit. Phosphoenolpyruvate carboxylase was reported increased under various conditions leading to gluconeogenesis (89). Pyruvate kinase, on the other hand, was reported decreased in gluconeogenesis by Krebs and Eggleston (35). The latter finding does not agree with the postulate of Weber.

Leloir and Goldemberg (38) reported that glycogen synthetase activity in vivo is probably regulated by the glucose-6-phosphate concentration in the liver. Steiner and Williams (65) however, observed low glucose-6-phosphate levels in fasting rats, and increased glycogen deposition upon insulin injection, without concomitant increases in liver glucose-6-phosphate concentration.

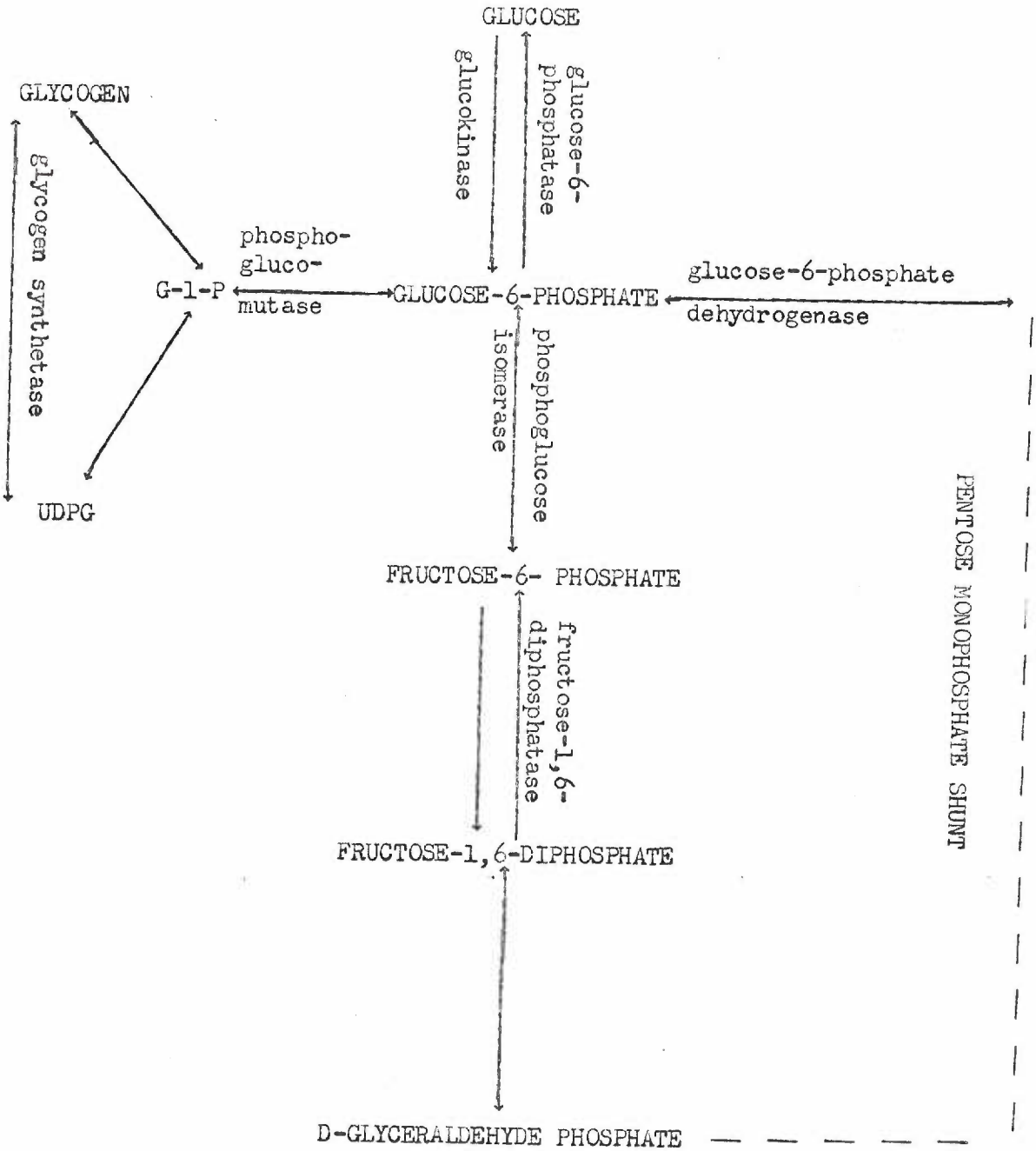
The study of the enzymes and conditions involved in hepatic gluconeogenesis indicates that some of the enzymes of this pathway have invariably increased activities during increased glucose synthesis. The activities of some of the key enzymes concerned with endogeneous glucose formation were assayed by Todd and coworkers[1]. Rats were fed semisynthetic, isocaloric diets for 24 hours, with or without supplementation by 10 percent glycine. Some of the animals fed the control diet and the glycine diet were sacrificed after 4 hours fast. These were called non-stressed animals. Other rats underwent a 90 minute stress consisting of three 10 minute swim periods in 14°C water, alternating with two half hour rest periods at room temperature. Stressed animals were sacrificed immediately following the last swim. Other rats were permitted to rest for three hours after the last swim. These latter were called recovered animals. Following the above procedure, rats in three different physiological conditions were obtained: non-stressed, stressed, and recovered. Both control fed and glycine fed rats were treated as described. The composition of the diets and the technique of the swim stress have been described by Todd and Allen (74).

The metabolic changes to be discussed and the enzymes involved in these reactions are depicted in Fig. 1.

[1] Todd, W. R., Harris, M., and Laastuen, L. Unpublished data, Univ. of Oregon Medical School, 1962.

Figure 1

Pathways of carbohydrate metabolism in hepatic tissue of the rat.


GLUCOSE
glucokinase
glucose-6-phosphatase
GLYCOGEN
glycogen synthetase
phospho-gluco-mutase
G-1-P
GLUCOSE-6-PHOSPHATE
glucose-6-phosphate dehydrogenase
isomerase
phosphoglucose
UDPG
FRUCTOSE-6- PHOSPHATE
fructose-1,6-diphosphatase
FRUCTOSE-1,6-DIPHOSPHATE
D-GLYCERALDEHYDE PHOSPHATE
PENTOSE MONOPHOSPHATE SHUNT

Fructose-1,6-diphosphatase activity increased[1] following both stress and recovery in the glycine fed animals. The enzyme activity did not increase in control fed animals under the same conditions. These results would be a clear indication of increased gluconeogenesis in the glycine fed stressed rats, had the activity of glucose-6-phosphatase, the other key gluconeogenetic enzyme assayed, not been decreased in glycine fed non-stressed rats (compared to the levels of this enzyme in control fed rats). The glucose-6-phosphatase activity did not increase upon stress, nor following recovery in the glycine fed rats, however, it decreased in control fed rats recovering from swim stress. Therefore, it can be said only that the level of glucose-6-phosphate activity is maintained in glycine fed rats in all three physiological conditions, whereas it is not maintained in the control fed rats, although the starting levels are higher. The gluconeogenetic enzymes, according to Weber (82) are maintained, and/or increased in all conditions leading to gluconeogenesis, including starvation.

Hepatic glucokinase activity[1] in the glycine fed recovered rat was lower than in control fed recovered rats. As previously mentioned, the activity of this enzyme was decreased in fasting rats (17).

[1] Todd, W. R., Harris, M., and Laastuen, L. Unpublished data, Univ. of Oregon Medical School, 1962.

Glucose-6-phosphate dehydrogenase activity was consistently elevated in the glycine fed rats[1] in all three physiological conditions. This finding is difficult to interpret. The hexose monophosphate shunt pathway was postulated (23) to help regulate glycolysis by controlling the nicotinamide adenine dinucleotide phosphate (NADP) supply of the cell. According to the postulate, an increase in the NADP/NADPH$^+$ ratio will suppress glycolysis and funnel glucose-6-phosphate into the shunt. The resultant 6-phosphogluconate accumulation inhibits phosphoglucose isomerase activity and causes accumulation of glucose-6-phosphate. This in turn inhibits glucokinase activity, hence inhibits glycolysis.

Another role of the pentose shunt is in ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) synthesis. Increased P^{32} uptake by hepatic RNA and elevated hepatic RNA levels upon glycine feeding have been reported by Munro and Mukerji (48). Regulation of fat synthesis by virtue of NADP reduction is another implied function of the hexose monophosphate shunt. However, the calculated theorethical rate of this metabolic pathway relative to other pathways utilizing glucose-6-phosphate is only about 2 percent (1).

From previous nitrogen balance studies in this laboratory (70) it is known that glycine fed rats retain less nitrogen under some

[1] Todd, W. R., Harris, M., and Laastuen, L. Unpublished data, Univ. of Oregon Medical School, 1962.

experimental conditions than do control fed rats. The more negative nitrogen balance of glycine prefed rats during fasting indicates that protein synthesis, and therefore enzyme synthesis may be impeded. Under conditions unfavorable for enzyme synthesis, as in fasting, only synthesis of essential enzymes is maintained or increased (27, 81, 82). From this reasoning fructose-1,6-diphosphatase and glucose-6-phosphate dehydrogenase both must be key enzymes in the liver of the glycine fed stressed and recovered rats.

Glucose-6-phosphate dependent glycogen synthetase activity was maintained[2] in the liver of the glycine fed recovered animal. In control fed recovered rats the activity of this enzyme was decreased to one third the non-stressed level. Glycogen synthetase mediates an irreversible reaction in the metabolic pathway between fructose-6-phosphate and glycogen according to Tarnowsky and coworkers (71). The maintenance of high levels of this enzyme in glycine fed recovered rats[2], where the liver glycogen levels are elevated, is clear evidence for increased glycogen formation, as opposed to the possibility of decreased glycogen utilization.

[2] Todd, W. R., and Laastuen, L. Unpublished data, Univ. of Oregon Medical School, 1965.

FEEDING EXPERIMENTS AND OXIDATIVE PHOSPHORYLATION

In the course of gluconeogenesis, amino acids are deaminated, oxidized, and funneled into the tricarboxylic acid cycle, to serve as substrates for both gluconeogenesis and oxidative phosphorylation.

No attempt is made here to discuss the mechanism involved in oxidative phosphorylation. Suffice to say that intact, isolated mitochondria contain all the components for the oxidation of citric acid cycle intermediates (37). Mitochondria are also capable of fatty acid oxidation. The electrons generated from the oxidation of the various substrates are accepted by the respiratory chain, an important component of the mitochondrial structure. This chain consists of electron carriers arranged in an increasing order of redox potential. Coupled to the electron carrier chain, unknown mechanisms conserve the energy of oxidation, ultimately in the form of adenosine triphosphate (ATP). For energy to be conserved however, the mitochondrial structure must be relatively intact. In the course of <u>in vitro</u> studies of the mitochondria, structural integrity, although visible under the electron microscope, is usually inferred on the basis of experimental results.

A preliminary study was made in this laboratory (30) on the effect of glycine feeding on isolated, disrupted rat liver mitochondria. The specific activity of reduced nicotinamide adenine dinucleotide (NADH) dehydrogenases was found to be increased by about 25 percent in hepa-

tic mitochondria isolated from stressed, glycine fed rats. Changes in oxidation rates alone have little bearing on in vivo conditions however, especially relatively small changes. The dehydrogenase activity in recovered, glycine fed rats' hepatic mitochondria did not differ from the activity of either the glycine fed non-stressed, or the control fed recovered animals. Yet it is in the livers of the glycine fed recovered animals, that the great increases in liver glycogen levels are observed.

Although there is abundant literature dealing with mitochondrial oxidative phosphorylation per se, only a handful of diet connected applications of such investigations have been reported. Considering the great complexity of mitochondrial functions and the interdependence of function and structure, interpretation of in vitro experimental results and correlation of these results with in vivo conditions is at best difficult.

Stripe and Schwartz (66) reported decreased $NADH^+$ dehydrogenase activity in hepatic mitochondria isolated from rats fed a Torula yeast containing diet. This yeast is an inadequate protein source.

Dietary deficiency of essential fatty acids affects uncoupling of oxidation from phosphorylation in hepatic mitochondria in such a manner as to be indicative of some "molecular defect" (86). The defect is postulated to be caused by the absence of essential fatty acids in the structure that determines the spatial relationship

between the electron transport chain and oxidative phosphorylation. Using whole liver homogenates from essential fatty acid deficient weanling rats, Smith and DeLuca (64) found the oxidation rates of citrate, succinate, α-ketoglutarate, malate, pyruvate, and caprylate increased, compared to tissue from animals fed essential fatty acids. Oxidation of β-hydroxybutyrate was unchanged. The fatty acid deficiency was induced in two ways: by feeding fat free diets, or by feeding diets high in saturated fat content. According to Ito and Johnson (31) there is no impairment of oxidative phosphorylation in preparations from the livers of fatty acid deficient rats. Aging, uncouplers, and inhibitors, however, have more pronounced effects on these preparations than on preparations from control fed animals. These results again indicate instability rather than impairment of the mitochondrial structures due to fatty acid deficiency.

The rate of mitochondrial respiration depends not only on substrate and oxygen supply, but also on the concentration of adenosine diphosphate (ADP), inorganic phosphate (Pi), and the end product of oxidative phosphorylation, adenosine triphosphate (ATP). ADP concentration determines respiratory rates of tightly coupled, structurally undamaged mitochondria (37). Respiratory control by ADP is defined as the rate of respiration in the presence of ADP divided by the respiratory rate in its absence.

Decreased respiratory control (56), decreased phosphorus to oxygen ratios (P/O ratios), and decreased oxidase activiti[illegible] were

reported in hepatic mitochondria prepared from niacin and riboflavin deficient weanling rats. Although not diet connected, it is of interest that rates of oxidative phosphorylation and of ATP synthesis were decreased in cardiac tissue with elevated glycogen levels (3). Hepatic ATP concentrations were reported to be decreased in rats fed a protein meal (14), compared to rats fasted 10 hours.

RELATION OF THE GLYCINE EFFECT TO AMINO ACID IMBALANCE

The possibility that enzyme synthesis is blocked to some extent, due to glycine feeding and stress, is consistent with changes (70) found in the nitrogen balance of glycine fed rats by Talman, in this laboratory. Rats were trained to consume liquid diets. A 24 hour fast was preceded by 48 hours of feeding the glycine or the control diets. The excess net nitrogen excreted by glycine prefed rats during the fast period was estimated to be equivalent to, or in excess of, the amount of nitrogen contained in 2 g of body tissue. Most of the excess nitrogen excreted was in the form of urea (70) and practically no unmetabolized glycine was excreted. Increased urea excretion may indicate dietary amino acid imbalance. Increased nitrogen excretion is of course also a manifestation of gluconeogenesis.

Livingstone (40) found lowered respiratory quotients in the glycine prefed animals upon fasting compared to control prefed rats.

Only after 11 to 13 hours fast did the respiratory quotients of control fed rats begin to decrease from the original value of 0.962. The decrease signifies that carbohydrate reserves had been depleted and oxidation of fat or protein was increasing. The difference in respiratory quotients between the control fed and the glycine fed animals was largest at 7 to 9 hours fast: 0.962 and 0.801 respectively. These results offer further evidence of greater utilization of other than carbohydrate stores for energy metabolism in the glycine fed rat than in the control fed rat. The difference in respiratory quotients is also consistent with increased glyconeogenesis in glycine fed rats from protein, i. e. from material with less oxygen content than carbohydrate.

Utilization of protein stores for energy metabolism must be at the expense of the amino acid pool available for protein synthesis. However, the selective nature of enzyme depletion, synthesis, and maintenance under conditions of protein depletion is well documented (27, 80, 81, 82). Klain and coworkers (32) reported increased hepatic transaminase activities upon the feeding of excessive amounts of leucine with an otherwise adequate ration. Klain also found decreased nitrogen retention (33) and increased liver glutamic oxalacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) activities in rats fed excess methionine.

A great deal of work has recently been published on the effects of dietary amino acid imbalance. In most of the published

work the main criterion of imbalance is the decline in growth rate of very young animals. Decreased nitrogen retention is another general symptom of this dietary condition. In those experiments where liver glycogen levels were measured, these were found to be elevated. Another common characteristic of dietary amino acid imbalance is the decreased appetite of the animals fed such diets (28, 32, 58, 59).

In this laboratory the glycine fed rats had decreased appetite, decreased nitrogen retention, and increased liver glycogen levels upon stress, and recovery from stress. Although most investigations of amino acid imbalance involve the excess or the lack of essential amino acids, there are a few imbalance studies indicating that dispensable amino acids may play a role in the dietary amino acid imbalance also. Beuer and coworkers (5) fed weanling rats a mixture of essential amino acids, and the following nonessential amino acids: alanine, aspartic acid, glutamic acid, asparagine, glycine, proline, and serine. Omission of glutamic acid or of proline from the diet significantly decreased the weight gain of the rats. Serine or glycine omission lowered weight gain to a lesser extent.

Harper and Rogers (28) distinguish between various categories of amino acid imbalances. Accordingly, in "true amino acid imbalance" one or more amino acids are present in the diet in limiting concentrations. When the concentrations of two amino acids are limiting

and an excess of the second most limiting amino acid is added to the diet, growth is retarded. Such relationship exists between lysine and threonine, in a ration with rice as the dietary protein source. Another example of an amino acid imbalanced diet is one containing 6 percent fibrin as the protein source. Fibrin is low in histidine. If such a ration is supplemented by a mixture of essential amino acids from which histidine is omitted, amino acid imbalance is produced. Growth retardation due to the feeding of this diet can be corrected by supplementation with histidine. Harper considers the interrelationship of dietary leucine, isoleucine, and valine as an antagonism, rather than imbalance, because there is no growth response to increasing the concentration of the most limiting amino acid, and because of the structural similarities of the amino acids involved. Distinction between the two categories seems rather tenuous, especially since the gross manifestations of either type of imbalance are identical. The mechanism of growth retardation and associated effects, on the other hand, has not been elucidated. The difference between the categories seems to be in the means of producing the imbalance, rather than in the effect produced. Other investigators do not restrict the definition of dietary amino acid imbalance in this manner (11, 47).

A third definition of dietary amino acid imbalance is "amino acid toxicity" (26, 28, 47). This term refers to specific effects of the excess of a single amino acid in the diet, however, there

is much overlapping in this category. An example is the comparison of three groups of rats by Klavins and Johansen (34). Control diet was fed ad libitum to one group of animals. Another group received amino acid imbalanced diet, and a third group consisted of rats fed control diet, but restricted in food consumption to that of the imbalanced group. The imbalanced diets contained either 4.5 percent methionine or 4 percent homocysteine. The weight losses in the imbalanced group and the group with restricted food intake were identical. Atrophy of acini in various glands seemed to be due to inanition, but other histological changes were specific for the amino acid imbalances.

Amino acid imbalances connected with methionine have been studied most extensively. Methionine excess and deficiency both cause imbalance. Peretianu and Abraham (54) found that excess dietary methionine retarded growth more if ingested simultaneously with the protein portion of the diet, than if the dietary protein was fed separately and methionine ingestion was delayed. The growth retarding effect of methionine was more pronounced with a high protein diet. The rats used in the experiments were pair fed.

Bressani (8) fed growing dogs a diet limited in tryptophan, lysine, methionine, isoleucine, and threonine. When this diet was supplemented with 270 mg of methionine or valine per gram of dietary nitrogen, the weight gain and nitrogen retention of the experimental

animals decreased. Additional supplementation of the rations with threonine or isoleucine prevented the effects of amino acid imbalance. The addition of all four amino acids (methionine, valine, isoleucine, and threonine) increased nitrogen retention.

Prosky and Wannemacher (55) found a slight decrease in the growth rates of rats fed glycine (1.28 percent) or guanidoacetic acid (0.7 percent) as a dietary supplement. These effects were overcome by the addition of 0.7 percent methionine to the diets. Klain and coworkers (33) however, did not find growth rates of rats depressed by feeding a 15 percent casein containing diet supplemented with either 2 or 4 percent glycine. The difference may be due to the fact that Prosky and Wannemacher used weanling rats, while Klain's rats weighed 180 to 220 g. Klain and coworkers found that feeding 4 percent methionine supplemented diets depressed the weight gain of rats. Addition of 4 percent glycine to the methionine supplemented rations ameliorated the growth depressing effects of methionine to a greater extent than did the addition of 2 percent glycine. Excess methionine also increased liver glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities (33). Glycine ingestion with the methionine containing ration reduced the GOT activity, but increased the GPT activity in the liver. Daily food consumption of the rats fed the 4 percent methionine supplemented diet was reduced to

almost one half that of the controls, however. Food consumption of the animals fed the basal diet with added methionine and 2 percent glycine was better, and the consumption of the methionine and 4 percent glycine supplemented diet was equal to that of the controls.

Decreased appetite itself seems to be a characteristic effect of amino acid imbalance (28), and some of the findings attributed to the amino acid imbalance are due to the inanition. Klain and co-workers (32) found that cold exposure (7°C) of rats induced voluntary increase in the consumption of amino acid imbalanced rations. When the excess amino acid was 5 percent leucine (in a ration containing 9 percent casein), both cold exposed rats and those maintained at room temperature lost weight, although the cold exposed rats ate 75 percent more food than those kept at room temperature. When other amino acids were used as dietary additions (to produce the imbalance), the cold exposed rats ate considerably more of the imbalanced ration than those kept at room temperature, and they also gained much more weight. The weight gain of these rats was less, however, than the gain of rats fed the control diet. The same investigators found that amino acid imbalance due to the omission of isoleucine and valine from the diet was corrected, if leucine was also omitted from the diet. Food intake itself, however, may be regulated by the amino acid composition of the diet according to Sanahuja and coworkers (59). These investigators used 10 percent and 15 percent gluten

fortified with lysine as the dietary protein. Rations were fed ad libitum to normal rats weighing 50 to 60 g, and to rats originally weighing 80 to 85 g, and protein depleted before the experiments. Rats given a choice between imbalanced and protein free diets, ate the protein free diet preferentially. Liver glycogen levels of rats fed imbalanced diets with threonine the limiting amino acid — were elevated to as much as 10 percent (59), despite decreased food intake of these animals compared to control fed animals!

Force feeding (62) of amino acid deficient diets caused weight loss, increased liver weight, and increased liver glycogen levels. The effects were more pronounced than when the same rations were fed ad libitum. The levels of free amino acids in the livers were unchanged in rats fed amino acid imbalanced diets.

Harper and coworkers (53) investigated the effect of variation in "digestibility" between proteins, on plasma amino acid concentrations. Rats fed diets containing casein had higher plasma amino acid concentrations than zein fed rats. After zein feeding a very low plasma lysine concentration was observed. The authors interpreted this lowered amino acid level as the result of free lysine being "drawn into the cells during stimulation of protein synthesis caused by the influx of amino acids from the intestine". Competition for transport as a possible factor is not considered, despite the recognition of "amino acid antagonism" as a type of amino acid imbalance by the same group of investigators.

AMINO ACID ABSORPTION AND INTESTINAL TRANSPORT

Orten (51) found that individual amino acids were absorbed at different rates from different amino acid mixtures in a Thiry loop, formed in the small intestine of a human subject as a result of unfortunate circumstances during intestinal surgery. The presence of nonessential amino acids in the mixture retarded absorption in general and influenced absorption of specific amino acids in such a way that the order of absorption rates changed. Relative absorption rates of individual amino acids were changed more when one amino acid was almost absent, or was present at an overwhelming concentration. Orten's work substantiates the results found by her, as well as by various other investigators in experimental animals.

Newey and Smyth (49) used sacs of everted small intestine from rats, as well as an in vivo procedure to study the intestinal transport of glycine and glycylglycine. Their results indicated that the amino acid transfer (from mucosa to serosa) involves two steps. The entry of glycine into the epithelial cells of the gut wall (mucosal transfer, the first of the two steps) seemed to be competitive with methionine.

Matthews and Laster (44) studied the relationship of transport rates, chemical structure, and mutual inhibition of five monoamino monocarboxylic acids, one of them the non-metabolized α-amino iso-

butyric acid (AIB). Everted segments of hamster small intestine were used to determine amino acid transport rates against a concentration gradient. At concentrations below 1 mM the transport rates increased with side-chain length. Increase in initial concentration resulted in increased transport rate, until a limiting rate — characteristic for each amino acid — was reached. There is a straight line relationship between the reciprocal of the initial amino acid concentration (1/s) and the reciprocal of the transport rate (1/v). This relationship does not shed light on the nature of the mechanism, it only demonstrates that a saturable step is involved. K_t is denoted as the amino acid concentration at which transport appears to be one half maximal. The values obtained for the constants are dependent on the experimental techniques employed and cannot be regarded as simple analogues of the corresponding terms in the Michaelis-Menten notation. These terms, however, can be used to compare amino acid transport under standard conditions. The values for K_t decrease with increasing side chain length, except in the case of AIB; and so does the maximum apparent transport velocity (V_{max}). K_t values were identical for glycine and for AIB (44). V_{max} was statistically not different for glycine, AIB, and alanine. Predicted inhibitory effects of one amino acid on the rate of absorption of another were shown to agree well with experimental values (43).

The rates of inhibition of amino acid transport depend on the concentrations at which the rates are measured (44). Transport of a given amino acid can be inhibited by one of higher K_t if the latter is present at high enough concentration.

Transport studies are hampered by metabolic changes undergone by the transported amino acid. This particular problem can be overcome by the use of model amino acids (2), such as AIB, which are transported, but not metabolized.

INTRACELLULAR AMINO ACID TRANSPORT

Another approach to the study of amino acid transport rates is the use of Ehrlich (mouse tumor) ascites cells, which metabolize amino acids very slowly. Except for the study of intestinal amino acid transport, most of the relevant information is on ascites cells.

At least two transport systems were postulated for the uptake of neutral amino acids by cells (52), because the relative ability of one amino acid to inhibit the uptake of another shows inconsistencies. Leucine inhibits amino acid uptake by Ehrlich cells in the following (decreasing) order: valine, methionine, alanine, glycine. The order of inhibition by alanine or by glycine is approximately the opposite. The two transport systems were designated leucine preferring or L-mediation, and alanine preferring or A-mediation systems. Glycine and AIB enter the cells linearly with

time and build up high concentrations inside the cells (20 times the outside level). Leucine uptake from the medium is slow and is not linear. These results are interpreted to be evidence for separate exit mechanism for leucine (fast escape rate), and glycine or AIB (slow escape rate). The mode of escape cannot be diffusion because large amino acid molecules escape very fast (52). Leucine, isoleucine, valine, and phenylalanine have high affinity for the L-mediation system. Alanine, glycine, serine, threonine, proline, asparagine, and glutamine have high affinity for the A-mediation system. Methionine has high affinity for both transport mechanisms. The two systems overlap to some extent. A number of other systems have also been postulated for amino acid transport in the Ehrlich ascites cell (52). A recent study utilizing N-methylated amino acids (13) revealed more clearly the overlap of transport routes for specific amino acid groups.

A study of the transport of neutral amino acids into human red blood cells (87) revealed these systems: a mediated transport used by most neutral amino acids, a low capacity transport system for glycine and alanine, and another system involved in the transport of amino acids with large hydrocarbon side chains.

Begin and Scholefield (4) concluded from studies on mouse pancreas that at least three distinct sites operate in neutral amino acid transport of this tissue. According to their postulate

competitive inhibition is possible between an amino acid which is not transported at a specific site, and the amino acid which is normally transported there. Methionine and valine do not share a transport site in mouse pancreas, whereas they do in ascites tumor cells. Other aspects of amino acid transport into tissue cells are the interrelations and mechanisms of amino acid efflux as well as the role of exchange diffusion, apparently not mediated by any specific mechanism (29). Inconsistencies in affinity sequences based on transport rates, intracellular accumulation, or competition for transport of amino acids known to utilize the same transport site, indicate that other, yet unknown factors will have to be considered in the final understanding of amino acid transport.

PLASMA AMINO ACID LEVELS IN DIETARY AMINO ACID IMBALANCE

Because of the lack of uniformity in results and lack of agreement in interpretation of the data on amino acid transport by the various investigators in the field, cogent explanations of findings in this area are difficult. High plasma amino acid levels may be due to the maintenance of high steady state levels in tissues (11), indicating overall elevated amino acid concentrations. The intracellular amino acid concentration however, could be considerably lower than the plasma amino acid concentration due to the competi-

tive nature of the amino acid uptake. The same kind of reasoning applies to low plasma amino acid concentrations. Since in either case tissue amino acid concentration may or may not be comparable to the plasma level, the plasma amino acid concentration should not be regarded as identical to the amino acid level to which the intracellular enzymes are exposed.

Amino acid transport antagonisms can be expected to be less specific than competition for enzyme activity (11) and, therefore, more far reaching in their effect on cellular metabolism by virtue of unbalancing cellular amino acid levels. Amino acids with high transport affinity may influence the exchange and the accumulation of other, non-antagonistic amino acids in tissue cells. Practically nothing is known about amino acid interrelationships at the cell level in the intact animal.

Peraino and Harper (53) compared the plasma amino acid levels of rats which were force fed zein, casein, or their respective hydrolyzates. They found lack of correlation between the rates of absorption of dietary amino acids, as measured by the amino acid concentrations in portal blood, and the quantity of amino acids in the ration. The difference was considered to be due to complex causes rather than to speed of digestion or absorption alone. These studies show that amino acids in casein are "more available" for absorption than those in zein, therefore protein digestibility is a factor

among others in plasma amino acid concentrations. The possibility exists that amino acid absorption from zein is depressed due to the high concentration (24 percent) of leucine in this protein.

Before practical techniques for the quantitative determination of individual amino acids were available, Christensen and coworkers (12) studied the effect of feeding selected amino acids on the intra-, and extracellular distribution of free α-amino nitrogen in guinea pig tissues. A single dose of the individual amino acid to be tested was fed after 12 hours fast by medicine dropper. The animals were sacrificed three hours after the amino acid feeding. The feeding of 10 mmoles per kg body weight of proline, histidine, glycine, or methionine caused elevation of the plasma amino acid nitrogen concentration. Plasma and liver glycine concentrations increased after glycine feeding. The intracellular to extracellular distribution ratios of free α-amino nitrogen, other than glycine or glutamine, decreased. On the basis of the experimental results it was postulated by these workers, that the capacity of cells for concentrating individual amino acids is limited, and that the amino acids show competitive inhibition for this mechanism.

Swendseid and coworkers (69) determined plasma amino acid contents of weanling rats fed experimental diets for four weeks. In these experiments a low protein (8 percent casein supplemented with methionine) diet was further supplemented by various sources

of nonessential nitrogen. The resultant growth rates were compared. Of particular interest are the plasma amino acid concentrations of rats fed 7.5 percent glycine supplemented rations. The concentrations of most of the essential amino acids in the plasma of these rats decreased. The concentrations of the plasma nonessential amino acids increased to more than twice the nonessential amino acid concentration in the plasma of control fed rats. The increase was mainly due to the high plasma glycine concentrations. The concentration of serine also increased in the plasma of the rats fed the glycine supplemented rations. Of the essential amino acid concentrations — expressed as μmoles per 100 ml plasma — arginine, lysine, and tyrosine decreased from 9.8 to a trace, 47.5 to 28.6, and 6.4 to 2.9 respectively. Change in plasma methionine concentration was only slight. As stated previously (33, 55), there is some evidence of antagonism between methionine and glycine when feeding amino acid imbalanced diets. Decrease in the ratios of essential to nonessential amino acids in the plasma of these animals (69) may partly reflect the composition of the diets fed. These changes may also be directly connected with the growth retarding effects of imbalanced diets. Stucki and Harper (68) fed diets containing amino acid mixtures equivalent to 7.5 to 15 percent protein. The amino acid mixtures were the sole source of dietary nitrogen. The rats' growth rates were not materially altered as

a result of changes in the ratios of essential to nonessential amino acids between 4.0 and 0.5 in the diets. However chickens were quite sensitive in this respect (67).

Sanahuja and coworkers (59) fed lysine supplemented gluten diets with threonine deficient and threonine containing amino acid supplements. They compared plasma amino acid patterns of the amino acid imbalanced (threonine free) and of the control fed rats. The feeding periods lasted two weeks. Threonine, the limiting amino acid of the diet, was decreased to about one third (from 10 to 3 μmoles per 100 ml plasma) in the plasma of the rats fed the amino acid imbalanced diet. Valine, leucine, and lysine concentrations were greatly increased. Similar results were obtained when the imbalanced diets were fed for only 24 hours (58). A homeostatic mechanism is postulated by the authors to curtail the desire for food when "the ratio of the plasma concentration of each indispensable amino acid to that of the one lacking in the amino acid mixture, reaches some critical level".

Harper (26, 28) considers plasma amino acid changes "the only clear cut positive evidence of altered amino acid metabolism" in dietary amino acid imbalance.

STATEMENT OF PROBLEM

The glycine effect consists of increased carbohydrate maintenance during stress and resynthesis of carbohydrate during recovery from stress, in rats prefed 10 percent glycine containing rations. The diverse topics discussed in the introduction have a direct relation to the glycine effect. Included in these are:

a) Enzymes of carbohydrate metabolism involved in increased gluconeogenesis, discussed in relation to the postulated increased gluconeogenesis due to the glycine effect.

b) Dietary influences on mitochondrial oxidative phosphorylation, considered relevant to the work presented in this thesis.

c) Attempts to link the glycine effect to various aspects of amino acid metabolism and dietary amino acid imbalance.

The aspect of the glycine effect investigated in the subsequent work consists of resynthesis and redeposition of liver glycogen in rats recovered from cold water swim stress. The various experiments were planned to test the postulate that the glycine effect is due to an increase in gluconeogenesis. However, attempts to indicate the nature of the mechanisms involved have been only partially successful.

Through a study of oxidative phosphorylation it was hoped to determine whether the utilization of tricarboxylic acid cycle intermediates is increased in hepatic mitochondria prepared from glycine fed rats. Tricarboxylic acid cycle intermediates are also intermediates of amino acid catabolism.

Due to implications of this investigation, further feeding experiments were designed to evaluate the glycine effect in terms of dietary amino acid imbalance.

M A T E R I A L S A N D M E T H O D S

ANIMALS

a) Feeding schedule

Male Sprague-Dawley strain rats weighing from 150 to 200 g were used. These animals were maintained for 2 days or longer on ground Purina Laboratory Chow diet, to accustom them to feeding out of containers. At the start of the experiment 17 g control diet was placed in each cage for 24 hours. Immediately following, 17 g of the appropriate experimental diet was given, for 20 hours. In some experiments a 24 hour fast preceded this feeding schedule. After the 20 hours feeding period, the last meal was divided in two portions, as denoted in table I. Part a) of the ration contained all the ingredients of the control diet except dextrin. The rats were permitted to eat 2 g of this ration for one hour. After this time the empty food containers were removed from the cages. One g of part b) was then dissolved in 2 ml water. In this portion glucose was substituted for dextrin to improve solubility. This portion was administered to the animals by gastric gavage. The composition of the diet and the feeding schedule have been described previously by Todd and Allen (74).

In some of the feeding experiments part a) was omitted, since the difference in the diets' composition due to the omission is

not great, and the animals were eating up to the time of stomach tubing. Originally the last portion of the diet was divided in order to ensure equal caloric intake.

b) Swim stress

Four hours after stomach tubing, the non-stressed animals were sacrificed. At this time the swim stress started for the other experimental and control animals. The technique of the swim stress has been described by Todd and Allen (74). The swim stress consisted of three 10 minute swim periods in 14°C water interrupted by two 30 minute rest periods at room temperature (25°C). The initial 10 minute swim period was followed by 30 minutes rest. A second 10 minute swim period followed the first rest period. After an additional 30 minutes rest, the rats were made to swim for the third (final) 10 minutes. During the two rest periods the rats were cold, wet, and shivering. The combination of exercise and cold constituted a severe stress of 90 minutes duration. The animals sacrificed immediately following the last swim period are designated "stressed" animals. Some rats were permitted to rest for 3 hours at room temperature following the last swim period. At the end of this 3 hours additional rest period these animals were sacrificed and they are called "recovered" animals. All animals to be sacrificed were anesthesized by the injection of 4 mg of Nembutal per 100 g body weight.

c) Diets

Table I includes the diets used in the various feeding experiments. The glycine diet differs from the control diet by the replacement of 10 percent dextrin with 10 percent glycine. These two diets are isocaloric. α-Amino isobutyric acid (AIB) containing diets were prepared by adding 10 percent of the amino acid to the control diet. This procedure would have ensured isocaloric food intake, had the rats consumed 10 percent more of the AIB containing diet than of the other rations. Consumption of the AIB diet was slightly less than the consumption of control diet. Each rat almost invariably consumed 17 g control diet, but only one half or less of the animals fed the experimental diets consumed this amount. Rats that ate less than 12 g of the ration were not used for the experiments. Experimental and control animals were matched as closely as possible with regard to body weight and food consumption. Some control fed animals were restricted to 15 g or to 12 g of ration in order to achieve matching food intake. During the summer and early fall voluntary food consumption of all animals decreased and feeding experiments had to be suspended, usually until late October. The experiments including gliadin and AIB feeding were conducted with a 24 hour prefast immediately preceding the 20 hour feeding period in order to improve food intake.

Table I

COMPOSITION OF DIETS

in percent by weight

		CONTROL RATION	GLYCINE RATION	AIB[1] RATION	GLIADIN RATION	PROTEIN FREE CONTROL RATION	PROTEIN FREE GLYCINE RATION	PROTEIN FREE AIB RATION
a	Casein	16	16	16	0	0	0	0
	Brewer's yeast[2]	10	10	10	0	0	0	0
	Salt mixture[3]	5	5	5	5	5	5	5
	Wesson oil	5	5	5	5	5	5	5
	Cod liver oil	2	2	2	2	2	2	2
b	White corn dextrin	54	44	54	59	80	70	80
	Glucose	8	8	8	8	8	8	8
	Glycine	0	10	0	0	0	10	0
	AIB	0	0	10	0	0	0	10
	Gliadin	0	0	0	21	0	0	0

[1] α-amino isobutyric acid
[2] Squibb
[3] Nutritional Biochemical Corporation (salt mix W)

HANDLING OF TISSUES AND METHODS

a) Preparation for liver glycogen determination

Livers removed from anesthetized animals were blotted and minced in a garlic press. Approximately 1 g samples were transferred into 2 ml 30% KOH in weighed centrifuge tubes. All this handling was done in less than 60 seconds. The weight of the aliquot was recorded and the glycogen was precipitated and hydrolyzed by the method of Good, Kramer, and Somogyi (22). Glucose was estimated by the glucose oxidase method of Salomon and coworkers (57) after making the hydrolyzed samples alkaline with a few drops of 40% NaOH.

The rest of the liver was immediately chilled in ice.

b) Isolation of mitochondria

For mitochondrial preparations at least 4 g of liver was required. The isolation medium of Blanchaer (6) was used for the homogenates. The medium contained 0.25 mole sucrose, 1 mole ethylenediamine-tetraacetic acid (EDTA) and 10 g of fraction V bovine plasma albumin per liter. The pH of the medium was adjusted to 7.4 by the use of color comparison standards. A Beckman model G pH meter was used for other pH determinations.

Glass homogenizing tubes, ground glass pestles, 25 ml graduated cylinders, testtubes, and stirring rods were kept cold over

crushed ice. The cold, minced liver was weighed and homogenized in an equal volume of the medium described with not more than 4 strokes of the pestle. The resulting homogenate was poured into a graduated cylinder. The homogenizer tube was washed twice. These washings were transferred to the graduated cylinder. The homogenate was diluted to 20% (1 g liver per 5 ml volume). Ten ml aliquots were transferred to 50 ml round bottom lucite centrifuge tubes and centrifuged at 4°C in a model PR2 International centrifuge for 7 minutes at 600 RPM. The supernates were decanted into identical centrifuge tubes and centrifuged at the same speed for 10 minutes. Supernates from this centrifugation step were transferred to 15 ml lucite tubes and centrifuged in the No. 296 high-speed centrifuge head for 15 minutes at 10,000 x gravity. The resultant samples were decanted and the supernates were discarded. The centrifuge tubes were inverted to let the "fluffy layer", consisting of a mixture of microsomes and mitochondria, run down the sides of the tubes. This was wiped off. If the remaining bottom layer contained red blood cells at this time, they were removed by a cotton tipped applicator stick. No attempt was made for quantitative recovery of mitochondria. Homogeneity of sample was the purpose of this method of preparation. The preparations were resuspended and considered to represent 20% liver homogenates. Two aliquot samples were pooled and centrifuged at approximately 20,000 x gravity for 15 minutes. The final preparation was resus-

pended in 0.25 M sucrose containing 1% albumin, to make 50% homogenates (1 g equivalent of original liver in 2 ml volume). The final suspending medium did not contain EDTA because interference of this compound with oxidative phosphorylation at some concentrations has been observed (84).

The suspensions of prepared mitochondria were kept over ice and were immediately used for oxidative phosphorylation studies. The Warburg flasks were ready to receive the samples by this time. Only in this manner was it possible to get reproducible results with the samples from recovered animals, which deteriorated rapidly. Samples from non-stressed animals were more stable.

c) Substrates for oxidative phosphorylation

The preparation of the substrates may be of special interest because Lynn (42), whose hepatic mitochondria preparations gave as high P/O ratios as the ones found here, claims that without rigidly following his mixing procedure in the substrate preparation, the P/O ratios attained were only of the conventional magnitude. The substrate preparation method employed here was — at first inadvertently — very similar to that described by Lynn. The basic medium was prepared at room temperature by combining the following solutions:

16 ml 0.1 M K_2HPO_4

16 ml 0.2 M glycylglycine buffer, pH adjusted to 7.5 with 1.0 N KOH

4 ml 3 mM cytochrome c

4 ml 0.15 M $MgSO_4$

Variations of this medium by the addition of the individual substrates, and for purposes of special studies are indicated in tables II and III. Triple distilled water was used in the preparation of all solutions, except the medium for the first homogenate.

Instead of zero time flasks, 25 ml beakers were used with one half the amounts of media and mitochondria used as for the flasks. This was done in order to preserve mitochondrial preparations for a maximum number of concurrent oxidative phosphorylation studies, since the preparations, especially those originating from recovered animals, deteriorated rapidly. The purpose of the zero time flasks was to measure the starting inorganic phosphate (Pi) concentrations. A number of such preparations in beakers were compared with contents of Warburg flasks at zero time. Identical results with regard to Pi concentration proved that it was not necessary to occupy space on the Warburg stand for this purpose. The large open surface of a beaker compared to the small volume of mitochondria and reaction mixture in it was sufficient to aereate samples as well as they were aereated by shaking in the closed Warburg vessels at 90 oscillations per minute. Proof of this was found by the addition of hexokinase to samples in beakers, in which case the Pi uptake was identical to that of the aliquots in Warburg flasks.

Table II

REACTION MIXTURES

prepared for oxidative phosphorylation experiments

	REACTION MIXTURES		
	A	B	C
	succinate containing	DL-β-hydroxy-butyrate containing	α-ketoglutarate and malonate containing
ml basic medium	10	10	10
ml 0.06 M ATP[1]	1	1	1
ml 40 mM KF[2]	1	1	1
ml 0.05 M NAD	0	1	1
ml 0.1 M malonate	0	0	1
ml 0.1 M succinate	6	0	0
ml 0.2 M DL-β-OH-butyrate	0	5	0
ml 0.1 M α-keto-glutarate	0	0	4
pH adjusted to	7.2	7.4	7.2

[1] ATP was omitted in the reaction mixtures prepared for respiratory control studies employing ADP

[2] KF was omitted in some reaction mixtures, as referred to in the experimental section

Table III

PREPARATION OF INDIVIDUAL WARBURG FLASKS

for oxidative phosphorylation studies

Added to the main compartments of individual Warburg flasks[1]	Flasks containing reaction mixture[2] A	B	C
1.8 ml of premixed reaction mixture	A	B	C
ml 0.25 M sucrose[3]	0.5	0.2	0.2
ml mitochondrial suspension	0.5	0.8	0.8

[1] Hexokinase was added to the sidearms previous to filling the main compartments of the flasks. Hexokinase purchased from Sigma Chemical Co. St. Louis,Mo. The enzyme,in ammonium sulfate suspension,was diluted with 0.25 M glucose to contain 100 Kunitz-McDonald units in 0.2 ml volume (equivalent to 8.6 International Enzyme Units). This volume of diluted enzyme was added to the sidearms. The sidearms were stoppered and the flasks were immersed in crushed ice before the additions to the main compartments.

[2] designations from Table II

[3] Substitutions for special studies were made by omission of equal volume 0.25 M sucrose,as follows:

a) 0.1 ml glycine stock solution was added to some Warburg flasks.This is referred to in the section on results.

b) 0.1 ml DNP solution was added to some Warburg flasks. This is referred to in the section on results.

d) Manometric procedure

After 7 minutes equilibration with stopcocks open, the contents of the sidearms (Pi acceptor) were dumped into the main compartments of the Warburg flasks. The water bath temperature was 29°C and shaking rate was 90 oscillations per minute. Total liquid volume in the flasks including the KOH in the center wells was 3.2 ml. The flask constants were calculated for this volume (77). Samples were permitted to respire for 20 minutes. The majority of the experiments were performed with the reading and recording of the manometer changes every 5 minutes, but as more flasks and manometers were used this procedure was found to be impractical, and only 10 and 20 minute readings were recorded. Immediately following the 20 minute reading and with as great haste as possible, the manometers were opened and removed from the water bath. Water from the necks of the flasks was quickly wiped off, and the flasks were detached from the manometers and plunged into crushed ice. To each main compartment 1 ml 10% trichloracetic acid was added, and each flask was gently twirled. This procedure stopped all enzyme action and deproteinized the preparations. The time lapse between the last reading of the first flask and trichloracetic acid addition to the last flask was about two minutes when 18 flasks were used at the same time. This was no more of a time difference than the time between the tipping in of the hexokinase into the

main compartment of the first and last flasks at the start of the experiment. To minimize possible errors due to improper timing, consecutive flasks were not duplicate samples. Aliquots of a single sample were distributed in random sequence.

CO_2 in the Warburg flasks was absorbed by 0.2 ml of 5 N KOH in the center well of the flask into which fluted filter paper squares were placed in such a manner that the edges protruded slightly. When larger volume (20-25) ml flasks were used, the volume of the KOH as well as the size of the filter paper squares used had to be increased in order to achieve a comparable absorption of CO_2 (by comparison of oxygen uptake results with the two different sized flasks using aliquots of the same mitochondrial preparation).

The volume of the samples (in the main compartment) after the addition of trichloracetic acid was 4 ml. The flask contents were poured into conical centrifuge tubes and centrifuged for 10 minutes at approximately 2000 RPM.

e) Inorganic phosphorus determination

For inorganic phosphorus (Pi) determination the method of Lowry and Lopez (41) was used. From each centrifuge tube 1 ml supernate was pipetted into individual 10 ml volumetric flasks. After the dilution of these samples 0.2 and 0.5 ml aliquots were used for color development.

f) Protein determination

Aliquots of 0.05 and 0.10 ml of each mitochondrial preparation were transferred into round bottom cuvettes for protein determination by the biuret method. At the same time the protein concentration, or rather the optical density reading (O.D.) at 540 mμ, of 0.2 ml aliquots of the albumin containing suspending medium was also determined. To the samples in the cuvettes 0.2 ml of 0.4% sodium deoxycholate solution was added. The samples were left to stand with the deoxycholate in order to facilitate solution of mitochondrial proteins. After about one half hour the volumes were diluted to 1 ml with distilled water. The biuret determination method of Weichselbaum (83) was employed. A standard curve was prepared on mitochondrial protein by nitrogen determinations using the micro Kjeldahl method. Non protein nitrogen concentrations of mitochondria samples were subtracted from the total nitrogen values of the samples used for the micro Kjeldahl determinations as described previously (30).

g) Determination of succinic dehydrogenase activity

One other substrate, besides those described, was used — for the determination of succinic dehydrogenase activity. The reaction mixture was that described in Umbreit's Manometric methods (77). The high activity of this enzyme was reflected in the volume of oxygen taken up. Due to the high activity, the sample concentrations were considerably reduced by dilution with distilled water for this

determination. In the course of this experiment mitochondria were disrupted by the addition of $AlCl_3$ and $CaCl_2$ to the reaction mixture.

QUALITY OF THE ISOLATED MITOCHONDRIA

a) Respiratory control

Mitochondrial respiration rate depends on several factors as mentioned in the introduction. The idea that respiration of tightly coupled, structurally undamaged mitochondria is controlled by the availability of adenosine diphosphate (ADP) or an ADP generating system, is now generally accepted (18, 37). Besides ADP or an ADP generating system, H_2O in the presence of an uncoupling agent can also serve as phosphate acceptor (10, 19).

Respiratory control is defined as the ratio of the rate of respiration in the presence of a phosphate acceptor to the respiratory rate in its absence. Mitochondria prepared in the albumin containing isolation medium were compared to preparations in 0.25 M sucrose in preliminary experiments with respect to respiratory control. The latter preparations were unsatisfactory in this respect.

Mitochondria treated with an uncoupling agent may show decreased phosphorylation rates, or may lack phosphorylating ability completely. Decreased phosphorylation rates may also be due to aging of the preparations, or structural damage of the mitochondria by any other means. This phenomenon is called "loose coupling" (18, 37).

2,4-Dinitrophenol (DNP) is an uncoupler of oxidative phosphorylation. According to published results (19) 1×10^{-4} M concentrations of DNP uncouple oxidative phosphorylation and results in 2 to 3 fold increases of oxidation rates. 1×10^{-5} M DNP concentrations cause loose coupling (18).

DNP was used as uncoupling agent in several experiments. Using 0.1 ml stock solution, as described in table III, the DNP concentration in individual Warburg flasks was 1×10^{-4} M. These experiments were poorly reproducible and under our experimental conditions this DNP concentration caused loose coupling rather than uncoupling. Higher concentrations of DNP were not used partly due to solubility problems. The results did not warrant further investigation of the effects of uncouplers on hepatic mitochondria obtained from the various experimental animals.

b) The effect of fluoride addition

The activities of several samples were compared with and without fluoride addition. Most of the time there was only slight difference in the activity, using succinate as substrate. This was interpreted to mean that the structural integrity of the preparations was well maintained. Fluoride is an inhibitor of several enzymes, including adenosine triphosphatase, which is a latent mitochondrial enzyme, activated upon aging or otherwise damaging the mitochondria. An occasional preparation, however, did have higher P/O ratio in the presence of fluoride.

The effects of the addition of two fluoride concentrations, 4 μmoles and 20 μmoles, were compared. The higher fluoride concentration depressed the activities of the preparations, especially with β-hydroxybutyrate substrate; therefore the lower fluoride concentration was used routinely. Other investigators report similar results with fluoride (37, 45).

CALIBRATION OF MANOMETERS AND FLASKS

Manometers were calibrated with the help of a micrometer type calibrator using water. The flasks were first calibrated with mercury as described by Umbreit and coworkers (77). Later a faster method of calibration was developed using water. The flask to be calibrated was weighed, filled with distilled water, and weighed again. When it was attached to the manometer, if the water rose beyond the mark etched on the sidearm of the manometer, the manometer arm was carefully dried inside and outside. The flask was reweighed upon removing a drop of water and again attached to the manometer. This procedure was repeated until the water rose exactly to the mark on the sidearm. The weight of the water (previous weight) was then corrected for temperature and gave the volume sought. With a little practice this method was not only fast and accurate, but the very thorough dishwashing which is necessary after the use of mercury in the flasks was avoided.

HEPATIC ADENOSINE TRIPHOSPHATE ASSAY

For rapid sampling in the assay of adenosine triphosphate (ATP), the method of Schenker and O'Donnell (60) was followed, except that liquid nitrogen was found to be more suitable for immediate freezing of the samples than solid carbon dioxide. Within seconds of opening the abdomen of the rat, small pieces of liver were excised and dropped into a wide mouth, numbered thermos containing liquid nitrogen. For the assay a sample of approximately 200 mg was quickly weighed, and triturated in frozen 5% perchloric acid in a mortar over a slab of dry ice, until homogeneous. Samples were left for at least one half hour in the refrigerator. Samples were neutralized to slightly alkaline pH with KOH, and diluted 10 to 30 times. The suspension was centrifuged at approximately 3000 RPM for 20 minutes at 0°C. ATP concentrations of the resulting supernates were determined using a luciferase-luciferin extract (61). For each ATP determination 5 mg desiccated firefly lanterns were used. The enzyme-substrate extract was prepared according to the method of Seliger and McElroy (61), except that the dry lanterns were ground in several small volumes of buffer, instead of using the total volume all at once. This change permitted faster and more efficient extraction of enzyme and substrate. The final extract was stable for at least one week under refrigeration.The frozen liver samples were used within 2 hours of sacrifice because storage was found to cause a slow loss of ATP content. Each time ATP concentrations were measured, an ATP standard curve was prepared using the disodium salt of this compound. the

standard curve was constant for identical concentrations of the luciferase-luciferin extract prepared from the same shipment. Fluorescence was measured at 562 mμ wavelength using an Aminco - Bowman fluorimeter with the inciting light shut out. Addition of 20 μg glycine to samples to be assayed did not interfere with the assay.

PLASMA AMINO ACID ANALYSIS

Plasma from control fed and glycine fed non-stressed rats was analyzed for acid and neutral amino acid content. Blood was withdrawn by cardiac puncture from rats anesthetized in the usual way. Siliconed, heparinized syringes and needles were used. Rats weighing 160 g, fed the control or the glycine diet for 24 hours, were sacrificed without fast. Most, but not all of the rats had small amounts of food in their stomachs at the time of sacrifice. Clear, hemolysis free plasma was centrifuged at 3000 RPM one half hour at 4°C to remove suspended material, and 0.1 ml plasma was pipetted into 0.2 N citrate buffer at pH 3.25. The samples were stored frozen until used. A Beckman - Spinco model 120 amino acid analyzer was used[1].

STATISTICAL ANALYSES

Statistical analyses were done by the Student t test. Only differences of $p < 0.05$ or better were considered significant.

[1] the analytical method was made available by Jones, R. Personal Communication. 1966.

RESULTS

STRUCTURAL INTEGRITY OF THE HEPATIC MITOCHONDRIA

For the assay of oxidative phosphorylation rates, hepatic mitochondria from non-stressed and from recovered animals were used. Mitochondria in livers of stressed and recovered animals are swollen, as seen on slides of haematoxylin stained liver sections. Electron micrographs of hepatic mitochondria sampled from animals under various conditions of stress also show swelling, distortion, and loss of structural detail (86). Structural disorganization due to stress is assumed to be responsible for the greater variation in oxidative phosphorylation results when hepatic mitochondria from recovered rats were used.

Structural integrity of respiring mitochondria is indicated by the tightness of coupling (37). Respiratory control, using ADP as phosphate acceptor and succinate or α-ketoglutarate substrates, was between 1.4 and 2.2. The results after the addition of DNP to fresh mitochondria were variable, when the substrate used was succinate. Sometimes respiration was completely inhibited, in other instances the P/O ratios were greatly decreased. Often phosphorylation was completely prevented without any significant increase in the rate of respiration. With β-hydroxybutyrate as substrate, respiration was considerably stimulated by 1×10^{-4} M concentra-

tions of DNP. The results of the inhibition studies are summarized in table IV. Under the prevailing experimental conditions, 1×10^{-4} M DNP concentration in the media of respiring preparations of hepatic mitochondria from rats does not uncouple oxidative phosphorylation completely. "Loose coupling", similar to these results, has been reported by Ernster (18), using $1x10^{-5}$ M DNP. The greater resistance of these preparations to uncouplers seems to be due to the protection afforded by the albumin content of the isolation and suspension media (84, 85).

Fresh preparations of mitochondria gave consistently very high P/O ratios. Delay in the experiments (aging of the mitochondria due to delay) not only decreased P/O ratios, it also decreased the activities of the preparations. Still another indication of the structural stability of the freshly isolated mitochondria was the finding that omission of NAD from the media did not alter the oxidative phosphorylation rates using β-hydroxybutyrate substrate and hepatic mitochondria from non-stressed rats. It is established that intact mitochondria do not oxidize externally added $NADH^+$ (37).

There are great differences in reported respiratory control ratios in the literature, as determined by the manometric technique. When the substrate is succinate, respiratory control is known to be low compared to $NADH^+$ dependent substrates (18). Cereijo-Santalo (10) studied respiratory control with various

substrates at different concentrations using preparations of mouse liver mitochondria. These preparations did not show respiratory control with 20 μmoles fumarate, nor with 20 μmoles β-hydroxybutyrate substrates. With 20 μmoles α-ketoglutarate respiratory control was 1.7 when either ADP or DNP were used in the experiments. DNP was found to inhibit respiration at high concentrations, and also at low substrate concentration. This author also found that the DNP or ADP concentration necessary for maximal respiratory stimulation is higher, the more dehydrogenases are involved in the substrate oxidation.

Since in the absence of phosphate acceptor, the respiratory carriers are maximally reduced, the initial response upon addition of a phosphate acceptor will be immediately maximal. This may explain the higher respiratory control ratios (37) obtained in experiments using the oxygen electrode, to measure oxygen uptake. The respiration in such experiments is followed for only a few seconds.

Figure 2

Schematic representation of the electron transport chain and phosphorylating sites of mitochondria.

and thus obtaining a one step reaction with this $NADH^+$ requiring substrate. Low reaction rates and poor reproducibility with this substrate caused these experiments to be discontinued. β-hydroxybutyrate proved to be a more satisfactory substrate for the study of $NADH^+$ mediated reactions. In isolated mitochondria the enzymes required for the further oxidation of oxaloacetate are absent. Thus a single $NADH^+$ requiring oxidation step can be studied by using β-hydroxybutyrate as the substrate, without the necessity of using an inhibitor (36).

Results summarized in table V show that the differences between the rates of oxidative phosphorylation by mitochondria from glycine fed vs. control fed non-stressed rats are significant only with succinate used as substrate. The variability of α-ketoglutarate oxidation by individual mitochondrial preparations is most likely due to the low reaction rates of the preparations with this substrate, and to the involvement of two additional reactions, malonate inhibition and a substrate level phosphorylation. The ultimate electron acceptor in the oxidation of α-ketoglutarate is NAD, and the oxidation results in the formation of 3 ATP. Succinyl CoA, the product of α-ketoglutarate oxidation, undergoes a complex phosphorolytic cleavage, resulting in the formation of an additional ATP. This is the so called substrate level phosphorylation. The total number of ATP produced, when α-ketoglutarate is the substrate for oxidative phosphorylation, is thus four. It was felt

that performing a larger number of experiments might not lead to significant differences in the results between glycine fed and control fed animals due to the complexity of the reactions involved.

With β-hydroxybutyrate as the substrate, interpretation of the results as the equivalent of $NADH^+$ oxidation is probably not valid. The substrate itself is not involved directly in the tricarboxylic acid cycle. Changes in tricarboxylic acid cycle intermediates due to, or preceding gluconeogenesis will therefore not influence the activity of β-hydroxybutyric dehydrogenase. Indications of mitochondrial compartmentalization (37) raise the question of whether $NADH^+$ utilized in the β-hydroxybutyrate to oxaloacetate oxidation is all or part of the mitochondrial NAD pool. If it is only a part, is it of the same compartment as the NAD involved in the tricarboxylic acid cycle oxidations? In a way, however, the use of β-hydroxybutyrate as substrate was a fortunate choice. This will be discussed later. Results from preliminary experiments (30) in this laboratory, using disrupted mitochondria show identical $NADH^+$ dehydrogenase activities in hepatic mitochondria of glycine fed and control fed rats ; 2.29 and 2.30 μmoles $NADH^+$ dehydrogenated per minute, per mg mitochondrial nitrogen respectively. These results indicate that $NADH^+$ dehydrogenase activity itself is not rate limiting.

The difference in β-hydroxybutyrate oxidation between preparations from control fed and glycine fed rats is not significant

statistically and could be due to a difference in oxidative phosphorylation rate at one of the two sites, or at both sites of the electron transport chain common to both substrates, β-hydroxybutyrate and succinate. The results did not warrant further study of this possibility.

Succinate oxidation by preparations from control fed non-stressed animals proceeds at a significantly lower rate than oxidation by preparations from glycine fed non-stressed animals, as seen in table V. The results may indicate that the first phosphorylation site is not involved in changes caused by glycine feeding.

To establish whether the dehydrogenase involved is rate limiting under the conditions of the experiment, succinic dehydrogenase activities of preparations disrupted by the addition of $AlCl_3$ and $CaCl_2$ were determined. Oxidation rates of fresh phosphorylating aliquots were also measured. The results, in table VI indicate that succinic dehydrogenase activity is not rate limiting. Of the total dehydrogenase activity available, less is utilized during oxidative phosphorylation in the preparations from control fed animals than in preparations from glycine fed animals. In other words, more excess reserve enzyme activity is apparent in the mitochondria from control fed animals, since the dehydrogenase activities of the two kinds of preparations do not differ, but the coupled oxidation rates do. Since differences in the rates of oxidative phosphorylation of these preparations are due to glycine

feeding, the effects of in vitro glycine addition to mitochondria prepared from control fed animals was investigated. Data in table VII show that neither the rates of succinate oxidation, nor that of the phosphorylation connected with this substrate are influenced by the addition of 20 μg, or 200 μg glycine to the reaction mixture.

PHOSPHORUS TO OXYGEN RATIOS

The P/O ratios calculated from the oxygen and inorganic phosphate uptake rates presented in table V, are much higher than the generally accepted theorethical values. Conventionally, two phosphorylation steps are assigned to the passage of two electrons through the electron transport chain from succinate oxidation, and three phosphorylation steps from the oxidation of $NADH^+$. Recently several investigators reported phosphorylation efficiency of isolated heart sarcosomes (63), and hepatic mitochondria (42, 25), as high as the presently reported results. Smith and Hansen (63) found P/O ratios of 6 with pyruvate substrate, and P/O ratios of 4 with succinate substrate, using isolated heart mitochondria. Using liver mitochondria isolated from rats, Lynn (42) found P/O ratios approaching 7 when α-ketoglutarate substrate was used, and P/O ratios around 4 with succinate substrate. The preparation procedures used by this author were almost identical to those used in the present study. Lynn measured glucose-6-phosphate concentrations besides measuring the disappearance of inorganic phosphate

from the media; thus actual ATP formation was measured. The results indicate twice the phosphorylating efficiencies originally proposed for oxidative phosphorylation. The passage of one electron rather than that of two, through the respiratory chain, may thus lead to the formation of 3 ATP, as proposed by Boyer (7).

Because of confusing and conflicting reports on P/O rates, Lenaz and Beyer (39) analyzed the factors involved in the evaluation of oxygen uptake by manometric methods. According to their results underestimation of oxygen consumption is a common error due to inadequate shaking rates, incomplete CO_2 absorption, and slow initial equilibration of CO_2 between the gas and liquid phases. None of these factors is believed to be involved in the results presented here. It seems possible that the high concentration of bovine albumin present in the suspending media throughout the isolation procedure maintains a degree of mitochondrial structural integrity not realized through other isolation procedures. This resultant high phosphorylating efficiency could be responsible for the high P/O ratios. The stability of similar preparations as far as resistance to aging is concerned is also reported by Weinbach (84). These considerations do not seem to be of primary concern here, since the results reported are intended to compare activities of hepatic mitochondria isolated under identical conditions from control fed and glycine fed animals. With succinate as substrate, hepatic mitochondria from glycine fed non-stressed

animals show 21 percent higher oxygen utilization rates, and 35 percent higher phosphorus uptake rates than do identical preparations from control fed non-stressed animals.

There is, as mentioned in the introduction, evidence to indicate that some dietary deficiencies cause structural instability of hepatic mitochondria (86, 31). Such defect was detected only by using uncouplers, or by other means of weakening the link between oxidation and phosphorylation. Glycine feeding could conceivably have some beneficial effect on structural stability of mitochondria, since glycine constitutes 15 percent of the amino acids of mitochondrial structural protein (15), the largest single component on a mole per mole basis. In table IV, data from experiments designed to detect such differences of structural stability, are summarized. As indicated previously, DNP addition to respiring mitochondria resulted in "loose coupling" in the various preparations. Respiratory stimulation by DNP was apparent when the substrate was β-hydroxybutyrate, but not when it was succinate. Although more phosphorylating capacity was retained by mitochondria from the livers of glycine fed rats, when the substrate was succinate, the difference is not significant statistically, due to the poor reproducibility of the results in these experiments.

Table IV

INHIBITION OF OXIDATIVE PHOSPHORYLATION

by 1×10^{-4} M 2,4-dinitrophenol

Hepatic mitochondria prepared from rats fed control diet or glycine diet, and not stressed. Q_O and Q_{Pi} expressed as μ atoms uptake/mg protein/hour.

Substrate	Diet prefed	Q_O	Q_{Pi}	P/O
β-hydroxy-butyrate	control (9)[1]	0.89±0.06[2]	3.83±0.39	4.30±0.41
DNP added[3]	control (6)	1.57±0.15	2.40±0.64	1.60±0.39
β-hydroxy-butyrate	glycine (9)	1.05±0.08	4.26±0.29	4.07±0.18
DNP added	glycine (6)	1.53±0.17	1.93±0.37	1.45±0.54
succinate	control (12)	2.48±0.12	8.79±0.56	3.54±0.13
DNP added	control (8)	2.58±0.23	4.91±0.69	1.90±0.24
succinate	glycine (12)	2.99±0.16	11.87±1.01	3.96±0.17
DNP added	glycine (8)	2.72±0.23	7.22±1.04	2.56±0.31

[1] number of animals used
[2] standard error

[3] 2,4-dinitrophenol

Table V

OXIDATIVE PHOSPHORYLATION RATES

Hepatic mitochondria prepared from rats fed control diet or glycine diet, and not stressed. Q_O and Q_{Pi} expressed as μ atoms uptake/mg protein/hour.

Substrate	Diet prefed	Q_O	Q_{Pi}	P/O
β-hydroxy-butyrate	control (9)[1]	0.89±0.06[2]	3.83±0.39	4.30±0.41
	glycine (9)	1.05±0.08	4.26±0.29	4.07±0.18
succinate	control (12)	2.48±0.12	8.79±0.56	3.54±0.13
	glycine (12)	2.99±0.16[3]	11.87±1.01[3]	3.96±0.17
α-ketoglu-tarate with malonate	control (4)	0.51±0.10	3.28±0.40	6.42±0.84
	glycine (4)	0.60±0.07	3.44±0.43	5.75±0.41

[1] number of animals used
[2] standard error

[3] significantly different from control fed, $p \leq 0.02$

Table VI

SUCCINIC DEHYDROGENASE ACTIVITY AND COUPLED SUCCINATE OXIDATION

Hepatic mitochondria prepared from rats fed control diet or glycine diet, and not stressed. Activity expressed as μ atoms O/mg protein/hour.

ACTIVITY	Control-fed[1]	Glycine-fed[1]	p[4]
succinic dehydrogenase[3]	4.99±0.44[2]	5.33±0.37	
oxidative phosphorylation Q_O with succinate substrate aliquots of dehydrogenase samples	2.90±0.16	3.36±0.12	< 0.05
percent activity increase upon uncoupling	72.0 ±0.5	58.0 ±4.2	< 0.01

[1] data from 6 animals
[2] standard error

[3] $CaCl_2$ and $AlCl_3$ used to uncouple reactions
[4] statistical significance of the difference

Table VII

OXIDATIVE PHOSPHORYLATION RATES

effect of *in vitro* glycine concentration

Hepatic mitochondria prepared from rats[1] fed control diet, and not stressed. Q_O and Q_{Pi} expressed as μ atoms uptake per mg protein/hour. Succinate used as substrate.

	in vitro additions		
	none	20 μg glycine	200 μg glycine
Q_O	2.89±0.23[2]	2.92±0.22	2.90±0.19
Q_{Pi}	11.86±1.29	11.52±1.34	11.72±1.50
P/O	4.05±0.14	3.89±0.20	3.98±0.33

[1] 6 animals were used
[2] standard error

OXIDATIVE PHOSPHORYLATION RATES OF HEPATIC MITOCHONDRIA ISOLATED FROM RECOVERED RATS

Succinate and β-hydroxybutyrate were used as substrates for respiring mitochondrial preparations isolated from recovered rats. As the results in table VIII indicate, comparison of mitochondria from control fed and from glycine fed recovered rats does not show statistically different rates of respiration with β-hydroxybutyrate substrate. There is a difference in the oxidative phosphorylation rates when the substrate is succinate. This difference is in the opposite direction from that of the non-stressed preparations, as a survey of table IX will indicate. Hepatic mitochondria from control fed recovered rats oxidized succinate at a higher rate than did mitochondria from glycine fed recovered animals. The data in table IX indicate somewhat larger standard errors and decreased statistical significance of the differences in preparations from recovered animals, compared to nonstressed values. These may be the result of the structural changes, microscopically seen as swelling, due to the stress undergone by these animals. P/O ratios, however, did not decrease significantly, contrary to expectation in the case of structural damage.

The enzyme activities of hepatic mitochondria preparations from rats fed the same diet, but in the non-stressed and the recovered condition, are also compared in table IX. Differences

in oxidative phosphorylation rates, when the results are compared in this manner, can be readily seen between preparations from non-stressed and recovered animals. Hepatic mitochondria from glycine fed rats utilized β-hydroxybutyrate at the same rates, whether the preparations were from non-stressed or from recovered animals. The rate of succinate utilization decreased in preparations from glycine fed recovered animals (compared to those from glycine fed non-stressed rats). In contrast, the rates of utilization of both substrates increased significantly in the preparations from control fed recovered animals compared to those from control fed non-stressed rats. These results indicated the possibility that hepatic ATP levels in the control fed recovered rats were more depleted than in the glycine fed recovered rats, and that the observed increased rate of oxidative phosphorylation serves to replenish high energy intermediates. On this basis it was felt that determination of hepatic ATP levels would be informative.

HEPATIC ADENOSINE TRIPHOSPHATE LEVELS

In the livers of glycine fed rats more ATP may be stored and thus available to the glycine fed recovered rat, than in the livers of control fed rats. Results of hepatic ATP measurements are summarized in table X. Liver ATP is so labile that speedy removal and immediate freezing of the tissue sample is a requisite to prevent hydrolysis. The difference in ATP concentrations in

the livers of the glycine fed non-stressed rats compared to control fed non-stressed rats is of a magnitude that can be expected from the higher rates of oxidative phosphorylation by hepatic mitochondria isolated from glycine fed non-stressed rats. The biggest single increase in the hepatic ATP levels was found in the control fed animals immediately following stress. The ATP levels in the livers of the glycine fed stressed animals also increased, but only slightly. In glycine fed stressed animals the increase amounts to 11 percent of the non-stressed level ; in control fed stressed animals it is 96 percent of the non-stressed level. In recovered rats, both glycine fed and control fed, the hepatic ATP levels are of the same magnitude.

The work seemed to be at an impasse at this point because the results of the oxidative phosphorylation studies did not aid in developing an explanation of the established increased carbohydrate reserves in glycine fed recovered rats. These results indicate that the glycine effect is accompanied by lack of mitochondrial enzyme response upon recovery from stress in relation to energy metabolism, as judged by oxidative phosphorylation despite large available liver glycogen reserves. These results considered together with relatively small increases of key gluconeogenetic enzyme activities in the liver of the glycine fed rats may indicate interference with enzyme synthesis.

What possible reason can there be for an inhibition of enzyme synthesis due to glycine feeding?

Table VIII

OXIDATIVE PHOSPHORYLATION RATES

Hepatic mitochondria prepared from rats fed control diet or glycine diet, and recovered from stress. Q_O and Q_{Pi} expressed as μ atoms uptake/mg protein/hour.

SUBSTRATE	DIET	Q_O	Q_{Pi}	P/O
succinate	control (13)[1]	2.90±0.22[2]	11.68±1.25	4.03±0.66
succinate	glycine (13)	2.34±0.18[3]	8.20±1.03[3]	3.51±0.56
β-hydroxy-butyrate	control (6)	1.25±0.09	5.58±0.72	4.45±0.48
β-hydroxy-butyrate	glycine (6)	1.15±0.11	4.44±0.40	3.86±0.41

[1] number of animals used
[2] standard error

[3] significantly different compared to control fed, $p < 0.05$

Table IX

OXIDATIVE PHOSPHORYLATION RATES

Hepatic mitochondria prepared from rats fed control diet or glycine diet, and not stressed, or recovered from stress. Q_O and Q_{Pi} expressed as μ atoms uptake/mg protein/hour.

SUBSTRATE	DIET	PHISIOLOGICAL CONDITION	Q_O	Q_{Pi}	P/O
β-hydroxybutyrate	control	non-stressed (9)[1]	0.89±0.06[2]	3.83±0.39	4.30±0.41
		recovered (6)	1.25±0.09[3]	5.58±0.72[3]	4.45±0.48
succinate	control	non-stressed (12)	2.48±0.12	8.79±0.56	3.54±0.13
		recovered (13)	2.90±0.22[3]	11.68±1.24[3]	4.03±0.66
β-hydroxybutyrate	glycine	non-stressed (9)	1.05±0.08	4.26±0.29	4.07±0.18
		recovered (6)	1.15±0.11	4.44±0.40	3.86±0.41
succinate	glycine	non-stressed (12)	2.99±0.16	11.87±1.01	3.96±0.17
		recovered (13)	2.34±0.18[4]	8.20±1.03[4]	3.51±0.56

[1] number of animals used
[2] standard error

[3] significantly different from non-stressed, same diet $p < 0.01$
[4] significantly different from non-stressed, same diet $p < 0.02$

Table X

HEPATIC ADENOSINE TRIPHOSPHATE LEVELS

Samples obtained from rats prefed glycine diet or control diet, and non-stressed, stressed, or recovered from stress. ATP concentration expressed as μg ATP/g liver.

PHYSIOLOGICAL CONDITION		CONTROL DIET	GLYCINE DIET
non-stressed	(9)[1]	183 ± 12[2]	248 ± 15[5]
stressed	(6)	358 ± 28[3,4]	278 ± 19[3,6]
recovered	(6)	266 ± 26	266 ± 42

[1] number of animals in group
[2] standard error

[3] significantly different from non-stressed, same diet $p < 0.01$
[4] significantly different from recovered, same diet $p < 0.05$

[5] significantly different from control fed, same physiological condition, $p < 0.01$

[6] significantly different from control fed, same physiological condition, $p < 0.05$

The first possibility considered was that the large excess of glycine in the alimentary tract retards absorption of other, essential amino acids which utilize the same transport mechanism. The excess glycine may also interfere with amino acid transport in tissues. Glycine could also have some specific inhibitory action on protein synthesis. To study whether this line of reasoning was worth pursuing, an amino acid analogue, α-amino isobutyric acid (AIB) was fed in the same manner as glycine was fed.

DIETARY AMINO ACID IMBALANCE

AIB has similar intestinal (44) and tissue transport (2) characteristics to those of glycine, yet is not metabolized in animals. In the initial experiments voluntary food consumption of the rats was decreased to less than half the usual food intake. In order to overcome this difficulty, and because only comparison of liver glycogen levels was contemplated, the rats were fasted 24 hours before the experimental diets were fed. This fast constitutes a stress and consequently the "non-stressed" liver glycogen levels in table XII are only relative to the following severe stress, but they cannot be considered to be true non-stressed levels. Food intake was excellent and comparable in all animals.

When it became apparent that AIB had a similar (if less pronounced) effect to that of glycine, the glycine effect was postulated to involve amino acid imbalance or toxicity. From previous work

in this laboratory (70) it is known that glycine prefed animals show a greater negative nitrogen balance during stress than do control fed animals. Increased nitrogen excretion is one manifestation of the condition known as dietary amino acid imbalance (26, 28, 47). Weight loss or reduced weight gain is the most often used criterion of this condition. Several ad libitum feeding experiments show that glycine feeding reduces the rate of weight gain in growing rats compared to the control fed animals. AIB feeding caused growth to stop. These results are summarized in table XI.

There are several means of producing amino acid imbalance. One approach is the feeding of incomplete proteins. In order to find out whether such an imbalance also elevates liver glycogen levels of rats under the conditions of these experiments, the biologically incomplete protein, gliadin was substituted for casein in the diet. The results in table XII show that gliadin feeding elevates liver glycogen levels more than any of the other diets. used. This holds under all three physiological conditions. The definition of, and distinction between the various amino acid imbalances have been reviewed in the introduction. Those investigators who did measure liver glycogen levels while experimenting with dietary amino acid imbalance, found the glycogen levels elevated (59, 62).

Table XI

WEIGHT GAIN ON VARIOUS DIETS

Rats fed ad libitum control diet, glycine diet, AIB diet, control diet with 1.5% tyrosine, or glycine diet with 1.5% tyrosine for one week.

DIET	average weight gain g/week
control (10)[1]	34.0 ± 2.9[2]
glycine (12)	22.0 ± 2.9[3]
AIB (6)	3.8 ± 4.8[3,4]
glycine with 1.5% tyrosine (6)	29.0 ± 3.7
control with 1.5% tyrosine	36.0 ± 5.0[4]

[1] number of animals used
[2] standard error

[3] significantly different from control diet
[4] significantly different from glycine diet

Table XII

LIVER GLYCOGEN

Rats prefasted 24 hours and fed the control diet, glycine diet, AIB diet, or gliadin diet. Some animals were non-stressed, others stressed, and others recovered from stress.

percent liver glycogen ± standard error

PHYSIOLOGICAL CONDITION	CONTROL DIET	GLYCINE DIET	AIB DIET	GLIADIN DIET
non-stressed	3.6±0.4 (7)[1]	4.1±0.5 (6)	4.0±0.3 (9)	4.4±0.3 (7)
stressed	2.9±0.3 (5)	3.1±0.3 (4)	2.4±0.2 (6)	4.6±0.2[2] (6)
recovered	0.8±0.3 (7)	4.3±0.6[3] (7)	3.5±0.4[3] (7)	4.6±0.3[3,4] (7)

[1] number of animals used
[2] significantly different from control, glycine, and AIB fed stressed values, $p < 0.01$
[3] significantly different from control fed, recovered values, $p < 0.01$
[4] significantly different from AIB fed, recovered values, $p < 0.05$

Table XIII

LIVER GLYCOGEN

Rats fed protein free or casein containing diets[1], containing supplements as indicated.

percent liver glycogen ± standard error

PHYSIOLOGICAL CONDITION	PROTEIN FREE DIETS			CASEIN CONTAINING DIETS	
	CONTROL	GLYCINE	AIB	CONTROL	GLYCINE
non-stressed[2]	3.6±0.5	3.6±0.4	3.4±0.4	2.8±0.3	3.7±0.3
stressed[2]	1.6±0.4	3.0±0.3[3]	2.5±0.5	1.3±0.3	3.1±0.3[4]
recovered[2]	0.2±0.1	3.4±0.1[4]	1.6±0.3[4]	0.4±0.2	2.9±0.4[4]

[1] for composition of diets see table I
[2] each value represents data from 5 or 6 animals

[3] significantly different from control diet, same physiological condition, $p < 0.05$

[4] significantly different from control diet, same physiological condition, $p < 0.01$

Another approach is to study the feeding of glycine in a protein free diet. This resulted, rather unexpectedly, in elevated liver glycogen levels upon recovery from stress. These data are presented in table XIII. AIB feeding in a protein free diet produced similar, but less marked differences. These results suggest that the site of the possible amino acid antagonism is other than the intestinal absorption site.

PLASMA AMINO ACID CONCENTRATIONS

To gain more evidence in determining whether glycine feeding is an amino acid imbalance, plasma amino acid concentrations of glycine fed and control fed rats were determined. The results of these experiments are presented in table XIV. The results indicate significant changes in plasma amino acid concentrations due to glycine feeding. The concentrations of most neutral amino acids decreased due to the glycine feeding, many of the changes are statistically significant. The first two peaks of the chromatogram traces in table XIV represent threonine, glutamine, asparagine, and serine. Separation of these four amino acids is not satisfactory on the particular column used to indicate the exact position of glutamine and asparagine. Because of this uncertainty, the first two values can only be regarded as consistent with expected decrease in threonine (68) and increase in serine concentration upon glycine feeding. Significantly decreased were the

plasma levels of glutamic acid, proline, alanine, isoleucine, and tyrosine. The level of plasma glycine is increased almost 15 times in glycine fed rats. There is no difference in the plasma levels of methionine between glycine fed and control fed rats.

Changed plasma amino acid levels may be the single most consistent finding in dietary amino acid imbalance according to Harper (26, 28). Supplementation of the imbalanced diet by a small amount of the most limiting amino acid will correct the imbalance as well as prove its existence. Results in table XIV indicate that tyrosine may be the most limiting amino acid in this instance of amino acid imbalance because the relatively low plasma tyrosine concentration is considerably decreased by 24 hours glycine feeding. Tyrosine supplementation of the glycine ration seemed to be a good approach toward finding the most limiting amino acid, although tyrosine is not an essential amino acid. The results of the feeding experiments to be described are summarized in table XV. A decrease in the glycine effect is noticeable when this ration is supplemented by 0.5 percent tyrosine. The supplementation of the glycine ration by 1.5 percent tyrosine abolishes the glycine effect.

The possibility that tyrosine feeding lowers carbohydrate reserves under the experimental conditions employed was investigated by the supplementation of the control ration with this amino acid. Somewhat unexpectedly, the liver glycogen levels in the rats fed the 1.5 percent tyrosine containing control diet were

elevated over those of the control fed rats in all three physiological conditions. Could it be possible that tyrosine supplementation of the control diet causes yet another amino acid imbalance?!

Included among the data in table XI are results of feeding experiments with tyrosine supplemented rations. The feeding of tyrosine supplemented control diet for one week did not influence the weight gain of the rats. Tyrosine supplementation of the glycine ration improved the weight gain of the rats compared to glycine fed rats, but this improvement is not significant statistically, due to individual variations (large standard errors).

Table XIV

PLASMA AMINO ACID CONCENTRATIONS

Rats fed glycine diet or control diet ad libitum

	μmoles amino acid/100 ml plasma ± standard error		
	CONTROL FED (4)[1]	GLYCINE FED (4)[1]	p[3]
Peak No.1[2]	95.7 ±13.0	57.0 ± 6.2	< 0.05
Peak No.2[2]	33.2 ± 2.9	75.7 ± 3.3	< 0.01
Glutamic acid	23.8 ± 3.4	14.8 ± 2.2	< 0.05
Proline	44.9 ± 5.6	25.7 ± 2.6	< 0.02
Glycine	21.4 ± 1.9	308.0 ±50.0	< 0.01
Alanine	85.1 ±13.0	42.5 ± 2.4	< 0.02
Cysteine	3.2 ± 0.7	2.5 ± 0.7	
Valine	24.6 ± 4.2	15.5 ± 1.3	
Methionine	7.2 ± 0.8	8.1 ± 1.1	
Isoleucine	12.5 ± 2.0	7.4 ± 0.5	< 0.05
Leucine	19.6 ± 3.3	11.2 ± 1.3	
Tyrosine	12.3 ± 1.9	6.0 ± 0.5	< 0.02
Phenylalanine	6.6 ± 1.2	4.4 ± 0.3	

[1] number of animals used

[2] glutamine and asparagine do not separate from serine or threonine on the chromatography column used. This is referred to in the section on results.

[3] statistical significance of the difference

Table XV

LIVER GLYCOGEN

Rats fed glycine diet or control diet, with or without tyrosine supplementation, as indicated. Some animals were non-stressed, others were stressed, and some were recovered from stress.

percent liver glycoge n ± standard error

PHYSIOLOGICAL CONDITION	CONTROL DIET	GLYCINE DIET	GLYCINE DIET WITH 0.5% TYROSINE	GLYCINE DIET WITH 1.5% TYROSINE	CONTROL DIET WITH 1.5% TYROSINE
non-stressed	2.7±0.3 (7)[1]	3.7±0.2[2,3] (8)	3.5±0.6 (2)	2.6±0.4 (6)	4.3±0.5[2,3] (4)
stressed	1.3±0.2 (6)	3.6±0.4[3] (7)	2.8±0.0[3] (2)	2.0±0.2[3,4] (6)	2.0±0.2[3,4] (4)
recovered	0.5±0.1 (7)	3.5±0.4[3] (7)	1.8±0.2[3,4] (4)	0.7±0.2[4] (6)	1.3±0.2[3,4] (4)

[1] number of animals used

[2] significantly different compared to glycine diet with 1.5% tyrosine, same physiological condition

[3] significantly different compared to control diet, same physiological condition

[4] significantly different compared to glycine diet, same physiological condition

D I S C U S S I O N

Mitochondrial oxidative phosphorylation rates in preparations from the livers of glycine fed rats were found to be different from those from control fed rats.

In rats fed Purina Chow, Glick and Cohen (21) found that oxidation rates were increased in mitochondria isolated from livers obtained either at night, or following exercise. The P/O rates calculated from the activities measured in the nocturnal samples were elevated when the substrate used was α-ketoglutarate, citrate, or malate. P/O ratios were not elevated when succinate was the substrate. P/O ratios were unaffected by exercise.

Similar stress-connected increases in phosphorus and oxygen uptake were observed in this laboratory using isolated hepatic mitochondria of control fed rats; the changes did not influence the P/O ratios significantly, as the results in table IX show. In contrast to these observations, the oxidative phosphorylation activities of hepatic mitochondria isolated from glycine fed rats decreased or did not change following stress. The results may indicate that the capacity for oxygen and phosphorus uptake by hepatic mitochondria isolated from glycine fed rats cannot increase, or that there is no need for increased capacity in these animals. Without stress, however, hepatic mitochondria isolated from glycine fed rats exhibited higher respiratory activity, when succinate was the substrate oxi-

dized. The difference in the change of oxidative phosphorylation rates with respect to the substrate may have some metabolic significance. Succinate as a tricarboxylic acid cycle intermediate, may be less available for energy metabolism *in vivo* in the glycine fed recovered animal, due to its preferential utilization in gluconeogenesis. The enzyme activities concerned with oxidative phosphorylation could have adapted to the decreased substrate concentration by decreased *de novo* synthesis. β-hydroxybutyrate utilization does not depend on the availability of tricarboxylic acid cycle intermediates, yet the oxidation rate of this substrate by isolated hepatic mitochondria from glycine fed recovered rats —— while it did not decrease as in the case of succinate —— did not increase either. The lack of change in activity may indicate that hepatic mitochondrial enzymes in the glycine fed rat cannot adapt to the increased energy requirements of the stress, despite markedly elevated liver glycogen concentrations. The control fed rats did respond to the stimulus of the identical stress with increased hepatic mitochondrial oxidative phosphorylation capacity.

The conditions prerequisite to the increased gluconeogenesis in the glycine fed rats must be present in the non-stressed animal. The most reasonable explanation of the conditions prevailing prior to the gluconeogenetic stimulus (stress) is the availability of amino acids for deamination and oxidation. The presence of intermediary metabolites of amino acid catabolism leads to increased

concentration of tricarboxylic acid cycle intermediates. Since tricarboxylic acid cycle intermediates serve as substrates of oxidative phosphorylation as well as for gluconeogenesis, when gluconeogenesis does not require these intermediates, they are oxidized for energy. The lower respiratory quotient of glycine fed animals found by Livingstone (40) in this laboratory could be the result of increased conversion of amino acid carbon chains into carbohydrate (gluconeogenesis), or of increased utilization of the carbon chains for energy, which also requires elevated oxygen consumption and lowers R. Q. Data at hand do not allow a firm choice of one of these alternatives.

If glycine feeding thus channels amino acids into energy and carbohydrate metabolism, the amino acid pool available for protein synthesis is decreased. As a result, in the glycine fed recovered animal, increased synthesis of selected mitochondrial enzymes cannot take place, as in control fed animals. As the results indicate, enzyme synthesis may be actually decreased in mitochondria from the livers of glycine fed recovered animals if the observed differences in _in vitro_ enzyme activities are due to _in vivo_ adaptive responses to prevailing _in vivo_ substrate concentrations. This mechanism could be responsible for the unchanged rates of β-hydroxybutyrate oxidation by hepatic mitochondria isolated from glycine fed recovered rats (compared to glycine fed non-stressed rats). The increased utilization of β-hydroxybutyrate by mitochondria isolated from

control fed recovered rats' livers (compared to activities in preparations from control fed non-stressed rats), contradicts the assumption that there is no need for increased capacity for oxidative phosphorylation following stress. During recovery, oxidative phosphorylation should be a more important biological activity for the intact animal than liver glycogen deposition, unless blood sugar levels are higher than normal and also high energy intermediates are stored and become available in glycine fed rats upon stress.

The blood glucose concentrations of control fed and glycine fed recovered rats did not differ, yet these animals manifest the glycine effect by synthesis and redeposition of liver glycogen. Glycine fed recovered rats had five times as high liver glycogen levels as control fed recovered animals.

Hepatic adenosine triphosphate (ATP) concentrations were measured in glycine fed and control fed animals without stress, following stress, and after recovery from stress. The results, presented in table X, did not indicate storage of high energy intermediates in the glycine fed rats. Although of the non-stressed rats, the glycine fed animals had the higher hepatic ATP concentration, this was not maintained during stress. ATP levels in the livers of control fed stressed rats were far more increased than the ATP levels in the livers of glycine fed stressed rats. ATP levels in the livers of recovered rats did not differ as a result of ingesting the various diets.

Two kinds of interpretations seem possible from these results. One possibility is that more ATP is used during stress in the glycine fed animal than in the control fed animal. This consideration may be correlated with the available data on glucose-6-phosphate utilizing enzymes, if increased gluconeogenesis increases ATP requirements. However, it seems self-defeating to produce energy at the price of more energy utilization.

The second interpretation of the results of ATP analyses follows from considerations similar to those used in connection with the explanation of the oxidative phosphorylation results. ATP levels could increase in control fed stressed animals because enzymes for its production are synthesized or activated in response to stress. Enzyme synthesis in glycine fed animals may be blocked to some extent due to the utilization of amino acids for gluconeogenesis. The ATP concentration differences as well as the differences in oxidative phosphorylating enzyme activity may thus both be due to the same mechanism that accounts for the glycine effect.

A ten percent glycine diet can be considered an "amino acid excess", similar to other amino acid imbalanced diets described in the literature. Amino acid excess is reported to decrease growth rates and nitrogen retention of young growing animals. The results in table XI show that short term glycine feeding caused similarly decreased growth rates. Previous work in this laboratory (70) established that glycine prefed rats, upon fasting, retain less

nitrogen than control prefed rats. The question arose whether other amino acid imbalances can also cause increased synthesis and redeposition of liver glycogen upon recovery from swim stress.

When gliadin is the sole source of protein in the semisynthetic diet, amino acid imbalance is produced due to the inadequacy of lysine and tryptophan in the ration. Table XII shows that feeding of a gliadin diet to rats under identical conditions to the glycine feeding increases liver glycogen deposition. It is of interest to note that the gliadin diet caused the highest liver glycogen values and that these high levels were unchanged in all three physiological conditions.

α-Amino isobutyric acid (AIB) has the same intestinal transport characteristics as glycine, but is not metabolized in the animal body. It is, however, transported into tissue cells. The feeding of this amino acid analogue, at 10 percent of the diet (the same as in the case of glycine) also caused increased liver glycogen levels in rats recovered from stress. AIB feeding had somewhat less of an effect than glycine feeding. Since AIB is not metabolized, yet causes elevated liver glycogen levels, at least part of the mechanism of the effect must be due to transport antagonism, rather than to more specific metabolic antagonisms. The transport antagonism may be located at the site of the intestinal transport or it may consist of cell-transport antagonism, possibly both are involved. Yet if transport of other amino acids into liver cells were blocked

or reduced, the increased gluconeogenesis could not take place either. Hepatic gluconeogenesis could take place from precursors derived of amino acid catabolism, if the glycine feeding only delayed the transport of some essential amino acid so that protein synthesis is blocked. Such a mechanism would be conceivable if the intestinal transport of dietary amino acids, or their transport into the liver were delayed by glycine ingestion.

Munro and Mukerji (48), when studying changes in liver ribonucleic acid metabolism caused by the administration of a single dose of an amino acid in a protein free meal, found liver glycogen levels elevated following an 18 hour fast. The feeding of 1 g glycine increased liver glycogen levels threefold compared to glycogen levels of control fed animals. The feeding of similar amounts of methionine elevated liver glycogens fivefold, and leucine feeding caused a twofold liver glycogen increase. Comparison of the effects of protein free diets on liver glycogen levels were made in this laboratory with the results presented in table XIII. The feeding of 10 percent glycine in a protein free diet caused elevation of liver glycogen levels in the recovered animals compared to the recovered animals fed protein free control diet. AIB feeding in a protein free diet caused similar effects, though to a lesser degree. These results indicate that the transport or metabolic disturbance caused by glycine feeding is probably not at the intestinal amino acid transport site. The reason that this possibility cannot be

completely discounted though, is the magnitude of endogeneous protein secretion into the gut (88). In the case of rats on a protein free diet, it amounts to the equivalent of a diet containing 10 percent protein (76). Yet only a small part of the endogeneous secretion is lost in the feces (47), therefore constant reabsorption must be taking place. This absorption might be influenced by the presence of an excess glycine.

Christensen (11) has been the most consistent advocate of the theory of intracellular amino acid transport antagonisms. The study of amino acid accumulation in Ehrlich Ascites cells (52) yielded some insight into amino acid transport mechanisms, at least to the extent of differentiating between a leucine and an alanine preferring transport system for neutral amino acids. The transport systems overlap to some extent. There are also separate exit mechanisms postulated for amino acids leaving these cells, as discussed previously in the introduction.

The field of amino acid transport is not well understood. Also the basis for the adverse effects due to dietary amino acid imbalances has not been elucidated. The two mechanisms may well be related. If dietary amino acid excess causes delay in the transport of one or more essential amino acids to the site of protein synthesis, due to competition for the transport mechanism by the amino acid which is in excess, the results are predictable. The symptoms will be decreased protein synthesis, increased nitrogen

excretion, and increased utilization of the carbon chains, formed in amino acid catabolism, for other purposes than protein synthesis. These metabolic changes have been related to dietary amino acid excess, including glycine feeding.

Dietary amino acid imbalance due to the lack, or low level of one or more amino acids in the ration causes similar symptoms. The probable reason for this is that, as in the imbalance caused by amino acid excess, protein cannot be synthesized because one or more of the essential amino acids are missing, or are present in insufficient quantity at the site of protein synthesis.

Dietary amino acid imbalances have yielded altered plasma amino acid patterns in experimental animals. Christensen (11) points out that plasma amino acid levels cannot, or should not be interpreted as if these were the levels to which intracellular enzymes are exposed.

The results of plasma amino acid analyses, found in table XIV, show that many of the amino acid concentrations were changed due to feeding glycine for as short a period as 24 hours. The concentration of many of the amino acids transported by the alanine mediating mechanism (in Ehrlich ascites cells) were decreased in the plasma of rats fed the glycine diet. The concentration of basic amino acids was not measured since a separate transport system, less likely to be involved here, is postulated to be involved in their transport (13). Swendseid and coworkers reported decreased levels of

most essential amino acids in the plasma of glycine fed rats (69). Christensen reported elevated plasma levels of methionine, histidine, threonine, serine, and proline due to the feeding of glycine to rats for as short a time as three hours (11). Our results are similar to the results reported by Swendseid and coworkers (69), although the experimental conditions differed. The data in table XIV show significant decreases in the plasma concentrations of glutamic acid, alanine, isoleucine, and tyrosine. Of these amino acids only isoleucine is transported by the leucine mediating system in the Ehrlich Ascites cells (52). Isoleucine is an essential amino acid, and tyrosine exerts a sparing action on the essential amino acid, phenylalanine. The chromatographic technique used in the amino acid separation does not permit definite separation of several amino acids in the region of threonine and serine, as already explained in the experimental section. Therefore definite conclusions regarding the concentration changes of serine and threonine, due to glycine feeding, cannot be drawn from the appearance of the first two peaks (results summarized in table XIV). The results however, are consistent with increased serine and decreased threonine concentrations in the plasma of glycine fed rats. Due to the uncertainty regarding threonine concentrations, and the likelihood that isoleucine transport is not critically affected by an amino acid (glycine) which is mainly transported by the alanine preferring transport system, tyrosine, although not an essential amino acid, was chosen

as the first dietary supplement to be tried as an antagonist of the glycine effect. Another reason for chosing tyrosine was, that of the amino acids significantly affected by glycine feeding, this amino acid was present in the rats' plasma in the lowest concentration.

The results of feeding experiments, undertaken to test the theory that the glycine effect is due to an amino acid imbalance which can be corrected by the feeding of small amounts of the most limiting amino acid of the ration, together with the glycine ration, are summarized in table XV. Tyrosine, when fed at 1.5 percent concentration in the glycine diet, completely abolished the glycine effect! This result of tyrosine supplementation is not due to any specific effect on glycogen reserves, because when tyrosine was fed at the same concentration as with the glycine diet, but as a supplement of the control ration, liver glycogen levels were not lowered. Tyrosine supplementation of the control ration caused significant elevation of liver glycogen concentrations compared to those in rats fed control ration alone. This difference was noticeable in all three physiological conditions. A likely interpretation of the effect of tyrosine feeding on liver glycogen reserves is, that this effect is due to yet another imbalance caused by the excess tyrosine. Further proof for or against this hypothesis may be gained by increasing the tyrosine concentration in the diet.

The results of tyrosine supplementation of glycine ration and control ration strongly support the contention that the glycine effect is an amino acid imbalance and that the rate limiting amino acid is tyrosine. It may be of significance that neither glycine, nor tyrosine are essential amino acids, yet these two are involved in a dietary amino acid imbalance. The fact that these are nonessential amino acids may be the reason why glycine feeding causes less severe symptoms than in amino acid imbalances, in which essential amino acids are involved.

The results also indicate, but do not prove, that transport antagonism is the underlying mechanism of this, and probably of other amino acid imbalances, since there is no reason to believe that glycine shares enzyme sites in an inhibitory capacity with tyrosine. The fact that AIB feeding causes increased liver glycogen concentrations in stressed and in recovered rats is another indication of transport antagonism.

SUMMARY AND CONCLUSIONS

Previous work established that rats prefed a semisynthetic diet containing 10 percent glycine maintain liver glycogen and blood glucose at higher levels following a cold water swim stress, than do rats prefed isocaloric rations without added glycine. Glycine prefed rats permitted to recover for 3 hours following the swim stress have much higher liver glycogen levels than do the identically handled control prefed animals. The increase in carbohydrate levels of glycine fed animals following stress or recovery from stress is referred to as the glycine effect.

I. Hepatic mitochondria isolated from animals as indicated showed the following differences in oxidative phosphorylation rates:

1) Control prefed recovered rats compared to control prefed non-stressed rats:

a] succinate oxidation 17 percent increased
concomitant phosphorylation 32 percent increased
difference in P/O ratios not significant

b] β-hydroxybutyrate oxidation 40 percent increased
concomitant phosphorylation 45 percent increased

2) Glycine prefed recovered rats compared to glycine prefed non-stressed rats:

a] succinate oxidation 21 percent decreased
concomitant phosphorylation 44 percent decreased
difference in P/O ratios not significant

b] β-hydroxybutyrate oxidation and phosphorylation unchanged

3) The rates of succinate oxidation and concomitant phosphorylation were higher in glycine fed non-stressed rats than in control fed non-stressed rats.

II. Hepatic adenosine triphosphate (ATP) concentrations in glycine fed rats increased by 12 percent only following stress, while ATP concentrations under the same conditions almost doubled in the livers of control fed rats. The non-stressed hepatic ATP level of control fed rats was 36 percent lower than the corresponding ATP concentrations in glycine fed rats.

III. Decreased utilization of succinate by mitochondria isolated from glycine fed recovered rats could reflect adaptive response to in vivo decrease in the concentration of this substrate, due to increased gluconeogenesis. Likewise, increased rate of succinate utilization in preparations from non-stressed glycine fed rats, and higher ATP levels in livers of such animals may be adaptive responses to in vivo substrate concentrations. But stress-induced increased capacity for β-hydroxybutyrate utilization is missing in these preparations. Stress does not stimulate increased ATP synthesis in the livers of glycine fed stressed animals either, in contrast with the control fed animals where ATP synthesis increased in stress. At the same time carbohydrate reserves are higher in the glycine fed rats. Decreased enzyme protein synthesis may be the cause of these results. This could be possible if the glycine effect were a manifestation of amino acid imbalance.

IV. Feeding experiments were designed to study the connection between dietary amino acid imbalance and high liver glycogen levels. Feeding gliadin or α-amino isobutyric acid (AIB) supplemented diets caused elevated liver glycogen levels following recovery from stress. Both glycine and AIB, when fed in a diet devoid of other nitrogen source also caused elevation of liver glycogens in stressed and recovered rats.

V. AIB is transported, but not metabolized in the animal body; its effect on liver glycogen levels suggests a possible transport antagonism as the cause of this effect. The antagonism cannot be at the site of absorption since the effect is apparent with protein free diets also.

VI. Plasma samples from glycine fed and control fed rats were compared with respect to neutral amino acid concentrations. Many of the amino acids analyzed decreased in concentration in the plasma of glycine fed rats. Tyrosine and isoleucine, relatively low in concentration in the plasma of control fed animals, each decreased by approximately 50 percent following glycine feeding.

VII. Tyrosine supplementation of various diets showed:

a] when glycine diet containing 0.5 percent tyrosine was fed, a decreased glycine effect was observed

b] when glycine diet containing 1.5 percent tyrosine was fed, the glycine effect was abolished

c] when control diet containing 1.5 percent tyrosine was fed, liver glycogen levels were not decreased

VIII. Short term feeding experiments indicated that the glycine feeding caused a one third decrease in growth rates; tyrosine supplementation of the glycine diet improved the growth of the rats.

IX. Accepted proof for the existence of a dietary amino acid imbalance is:

a] changed plasma amino acid patterns

b] abolition of the imbalance by feeding small amounts of the most limiting amino acid with the imbalanced diet

X. The amino acid imbalance caused by glycine - tyrosine antagonism may be unique in that it involves two dispensable amino acids.

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